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WILLIAM H. TALIAFERRO, *Editor-in-Chief*

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EFFECT OF X-IRRADIATION ON HEMOLYSIN DECLINE

WILLIAM H. TALIAFERRO AND LUCY GRAVES TALIAFERRO

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Dougherty and White (1946, see also White and Dougherty, 1945), as a phase of their study of the pituitary-adrenal cortical control of the physiological activities of the lymphocyte, concluded that a part of the effect of X-irradiation on lymphocytes is mediated by this endocrine mechanism (reviews in White and Dougherty, 1946, Dougherty and White, 1947, and White, 1948 and 1949). These authors reported (1) that 200 r total body irradiation of CBA mice produced lymphopenia, tissue lymphocyte degeneration and increases in the total serum protein and γ globulin, (2) that a similar irradiation of intact and adrenalectomized mice, after their agglutinin titer to sheep cells had become low or undetectable, caused a nonspecific anamnestic response in both groups, and (3) that 10 r caused a similar response in intact but not in adrenalectomized mice. They, therefore, considered endocrine mediation necessary for anamnesis with small but not with large doses of X-rays.

Some of these general conclusions have been questioned (see reviews in Valentine et al, 1948, Craddock et al, 1949, and Taliaferro, 1949). In regard to a nonspecific anamnesis as a result of X-irradiation, Craddock and Lawrence (1949) failed to produce a significant rise in serum antibody of immunized rabbits irradiated with either 20 r or 250 r total body irradiation when H and O typhoid agglutinins and antisheep hemolysin had fallen to low levels. Similarly, Fischel et al (1949) failed to find a rise in circulating antibody nitrogen (using a quantitative precipitin method) following the administration of either adrenocorticotrophic hormones or 50 r of X-rays three months after the cessation of immunization with crystalline ovalbumin. These negative findings were obtained in spite of the fact that both agents induced a decrease in circulating lymphocytes.

The present experiments were designed to test further the ideas of White and Dougherty in regard to the direct release of antibody by large doses of X-rays given during antibody decline. Antisheep hemolysins in rabbits were used because antibody activity can be quantitatively determined and titrations can be closely enough spaced to evaluate deviations from the usual antibody course. The X-ray dose, generally administered twice, was 400 r, which is one-half the LD_{50}/30 days for rabbits (Bloom, 1948). A preliminary report of these results has been published by us (1949).

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The authors are indebted to Dr. P. P. H. De Bruyn of the Department of Anatomy of the University of Chicago for making available his unpublished studies on the effect of X-irradiation on the lymphatic tissue of the rabbit, to him and to Dr. E. L. Simmons and Mr. J. E. Trier on the staff of the Division of Biological and Medical Research of the Argonne National Laboratory for assistance in irradiating the rabbits and to Mrs. Dorothy W. Green for technical assistance.

MATERIALS AND METHODS

Our methods for maintaining the rabbits and injecting and standardizing the sheep red cells photometrically for immunization have been previously described (1950). Partial data on some of the rabbits have also been reported by us (1950) under identical numbers (see rabbits 1 to 8 and 14 to 17 in tables 6 and 7). For convenience the photometrically standardized red cell suspensions for immunization as described previously (1950) may be designated approximately as 1.25 ml 0.1% or 2×10^7 cells, 1.25 ml 1% or 2×10^8 cells and 1 ml of 4, 7 or 10% or 6.4×10^8, 1.1×10^9, 1.6×10^9 cells, respectively.

A hemolysin unit in this work is defined as the smallest amount of serum which produces 50% hemolysis of 1 ml of a standardized sheep red cell suspension in the presence of four 50% units of complement. Hemolysis was determined photometrically and determinations were based on plots of antibody against hemolysis according to von Krogh's equation, as already described by us (1950). Hemolysin units are given directly in the figures and are indicated as the dilution of antiserum contained in 1 ml, i.e., the reciprocal of the titer in the text.

Thin dried blood smears stained with May-Grünwald Giemsa were used for the differential blood counts.

Two rabbits were irradiated at a time in cellulose acetate boxes at about 77.5 cm from the target to the center of the animal. The X-ray machine was operated at 200 kilovolts and 15 milliamperes with 0.5 mm copper and 1.0 mm aluminum filters. Radiation was delivered at the rate of 14.5 to 15.5 r per minute as measured by a Victoreen chamber in air placed in the cages at the same location as the center of the rabbits. The beam was delivered horizontally and the cages were turned every 7 minutes to insure equal exposure. An electric fan was operated in the room to obviate overheating the rabbits.

EXPERIMENTAL RESULTS

Experiment 1.—The first experiment consisted of 8 rabbits, half of which (1 to 4) were injected into the marginal ear vein with 1.25 ml of 0.1% standardized sheep red blood cells per kg of rabbit and the other half of which (5 to 8) were similarly injected with 10 times the amount. Rabbits 1 to 4 and 6 to 8 weighed 2 to 2.7 kg. Rabbit 5 weighed 1.6 kg. Three and four weeks after the immunizing injection, 2 rabbits of each group (3 and 4, 7 and 8) were irradiated with 400 r. Nine and one-half, 11 and 12 weeks after immunization, all rabbits were injected intravenously with egg white at the rate of 10, 30 and 50 mg per kg rabbit, respectively. Ovalbumin* recrystallized three times was used for the first injection. After it elicited no appreciable anamnestic rise, a commercial egg white (Merck) was used for the other two. Both antigens elicited a strong precipitin reaction with their homologous antiserums. The rabbits were bled for hemolysin titrations once before immunization and in general twice a week for 14 weeks after immunization. White cell counts were made once a week.

Fifty percent hemolysin titers throughout the period of observation may be found in figure 1. Before immunization, hemolysin titers ranged from 1:1 to 1:40. These values are within the normal range reported by us previously (1950). Ten to 13 days after immunization, the hemolytic titer of the antiserums from three rabbits immunized with 1.25 ml of 0.1% red cells per kg rabbit reached peaks ranging from 1:2500 to 1:6300 and then declined logarithmically at a rapid rate for 1 to 2 weeks and more gradually thereafter. At one month, the titers ranged from 1:200 to 1:500 and at 14 weeks from 1:45 to 1:110. The titer of rabbit 4 was aberrant. It reached a peak of 1:400 after 10 days, decreased to 1:20 after 4 weeks and then began to rise gradually until it reached 1:350 during the 12th week and was 1:330 after 14 weeks.

The hemolytic titer of antiserums from the 4 rabbits immunized with 10 times the above amount of sheep red cells reached peaks ranging from 1:1800

* We are indebted to Dr. J. W. Moulder for preparing the ovalbumin.

to 1:4000 after 6 to 10 days, maintained a virtual plateau for about one week, decreased for three weeks, and two remained more or less stationary while the other two continued to decrease. At one month, the titers ranged from 1:210 to 1:350 and at 14 weeks from 1:80 to 1:250.

The declining trend in hemolysin titer (or ascending trend in rabbit 4) was not altered by two irradiations with 400 r 3 and 4 weeks after immunization.

Total leucocyte counts for three weeks before irradiation were normal (usually 9,000 or over per cmm). They had decreased to 600 to 1000 in all rabbits 5 days after the second X-ray dose, remained at this level for a week, and then increased to 2000 to 6000 in several days and to 6000 to 9000 in

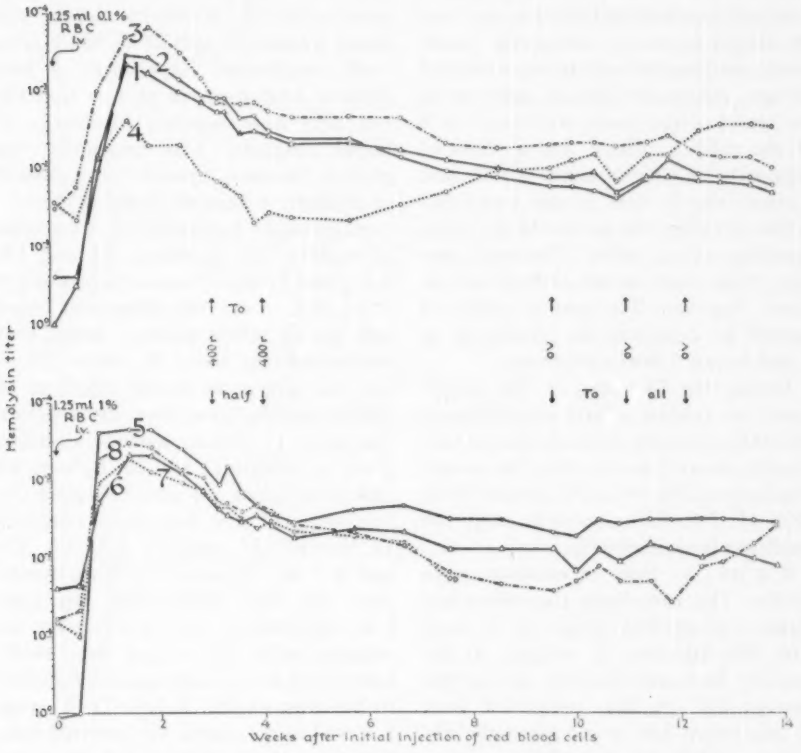

FIG. 1.—Hemolysin titer in photometric 50% units in 2 groups of rabbits before and after an intravenous injection of sheep red cells. Two rabbits in each group (broken lines) were irradiated with 400 r three and four weeks after immunization and all rabbits were injected with 10, 30 and 50 mg ovalbumin per kg rabbit 9½, 11 and 12 weeks after immunization.

Hemolysin had definitely increased in all rabbits on the 4th day, reached a peak on the 6th to 10th day and thereafter declined (rapidly at first and then more slowly except for a slight but steady increase in rabbit 4 from the 6th through the 14th week).

X-ray treatment did not appreciably affect the rapid decline of hemolysin and the ovalbumin injections possibly caused a slight irregularity in titer in rabbits 1, 2, 4, 5 and 6 and an anamnestic rise in rabbit 8.

another week. Seven weeks after the second dose, they had reached 9000 per cmm in all rabbits. All white cells decreased, especially lymphocytes. The total leucocyte count in the controls remained within a range of 8000 to 14,000 during this time. These data are similar to the results of Jacobson et al (1949).

Injections of egg white were made in an effort to ascertain whether X-irradiation had impaired or altered in any way the ability to elicit a nonspecific anamnestic reaction induced by an unrelated antigen. No marked change, however, in the trend of the curve was noted in 6 of the rabbits. There was a slight irregularity in titer of some rabbits and a slight rise in titer in one irradiated rabbit (8) after the second of the three injections of egg white. This may possibly have represented a slight anamnestic reaction. The rise in rabbit 4 cannot be described as anamnesis as it had begun 3 weeks previously.

During the 14 weeks of this experiment, the rabbits gained weight except for a temporary lag in the irradiated rabbits for about 2 weeks after the second irradiation. The majority gained 50 to 70% of their initial weight, and the smallest one gained 95%.

Results of this experiment were briefly: The hemolysin titer remained within the normal range for 3 days after the injection of antigen. It invariably increased sharply on the 4th through the 7th day, reached a peak on this latter day or increased slightly during a whole or part of the ensuing week, and then decreased—rapidly for 2 or 3 weeks and more slowly thereafter. During the rapidly declining phase, 400 r delivered to the whole body surface of the animal twice within one week caused no increase in hemolysin titer as measured by twice weekly titrations beginning one day after irradiation although the white cells, especially the lymphocytes, decreased to a low point a week after the second injection and the rabbits failed to gain weight normally. The injection of ovalbumin during the slowly declining phase may have caused a moderate heterologous anamnestic reaction in 1 of 7 rabbits.

Experiment 2.—In experiment 1, the first hemolysin titration after each irradiation was made at 24 hours. In experiment 2, a determination was made 6 hours as well as 24 hours after each irradiation. Experiment 2 also differed from experiment 1 in that the red cells were injected repeatedly in larger amounts. This treatment was given to increase, if possible, the amount of antibody within the lymphocytes.

Experiment 2 consisted of two groups of rabbits, one of which (12 and 13) was given 11 closely spaced injections of 1 ml of 4, 7 or 10% sheep red blood cells per kg rabbit within a period of 3 weeks and the other of which (14 to 17) was given an initial injection of 1.25 ml of 1% cells three weeks before the same 11 closely spaced injections given to rabbits 12 and 13. Rabbits 12 and 13 weighed 2.3 and 2.9 kg at the beginning of the injections. Rabbits 14 through 17 weighed 1.3, 2.0, 1.8 and 1.7 kg, respectively. Two weeks after the last immunizing injection, 5 of the rabbits (13 to 17) were irradiated with 400 r and two weeks later 3 (13 to 15) were again irradiated to the same extent. Rabbit 12 of group 1 served as a control. We omitted controls from the second group because we became convinced from experiment 1 that the antibody curve in irradiated animals was more important than the inclusion of unirradiated animals with their individual variation. Furthermore, we have since carried out an extensive study of hemolysin curves in multiply injected animals and have found the

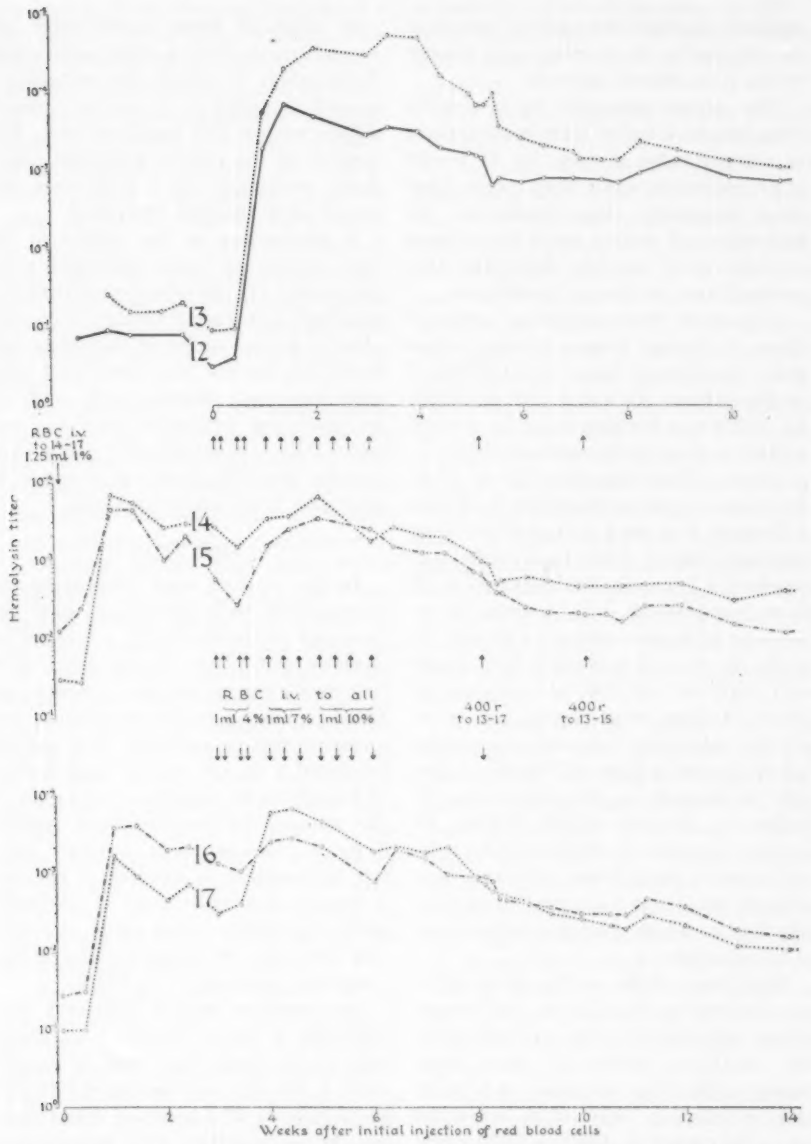

FIG. 2.—Hemolysin titer in photometric 50% units in 2 groups of rabbits before, during, and after immunization with a series of intravenous injections of sheep red cells. All rabbits (broken lines) except rabbit 12 were irradiated with 400 r two weeks after the last antigen injection and rabbits 13 through 15 were irradiated with 400 r two weeks later.

The course of hemolysin was similar to that in experiment 1 except that it remained high while antigen was being injected.

X-ray treatment did not appreciably affect the rapid or slow decline of hemolysin.

antibody decline after antigen injections are stopped to be in every way similar to the X-irradiated animals.

The rabbits were bled for hemolysin titrations once before immunization and in general twice a week for 14 weeks after immunization. They were bled more frequently after irradiation. All had white cell counts made three times a week for 1½ months beginning two weeks before the time of irradiation.

Data from these rabbits are given in figure 2. Before immunization, hemolysin titer ranged from 1:7 to 1:28 in 5 of the rabbits. It was 1:125 in rabbit 15, which is in the high range for normal rabbits as previously reported (1950).

After a single injection (14 to 17 in fig. 2) equivalent to that given to rabbits 5 through 8, a peak in hemolysin titer varying from 1:1500 to 1:6700 was reached in 7 days in 3 rabbits and in 10 days in rabbit 16. After a series of injections of larger amounts (12 and 13 in fig. 2) every 2 or 3 days, high peaks of 1:7000 to 1:37,000 were reached in 10 to 14 days, respectively, but when similar injections followed the initial injection after a lapse of 3 weeks in rabbits 14 through 17, the peak was only higher in the last rabbit. Rabbit 13 had an exceptionally high titer throughout immunization. When injections were stopped the titers decreased more rapidly for two weeks than thereafter—just as in experiment 1.

Irradiation of the rabbits with 400 r two and four weeks after the last immunizing injection of cells did not alter the declining hemolysin titer even though a titration was made at 6 hours after irradiation. The total leucocyte count decreased to 1000 to 2000/cmm three days after the first irradiation. It gradually rose to 2500 to 4500/cmm at the second treatment only to decrease slightly after 3 days. It then gradually rose although three weeks after the second treatment it was still subnormal. Even rabbit 17, which was not given a second irradiation, remained around 4000/cmm for this length of time. The weights of the rabbits remained essentially stationary for a little over two weeks after the first treatment.

A comparison of the rabbits in the four groups of both experiments indicate that (1) the initial peak titer was regained but not strikingly exceeded after a second series of injections and hemolysin decline was postponed until injections were discontinued, and (2) the declining hemolysin titer was not altered by irradiation with 400 r even though determinations were made as soon as 6 hours after treatment.

DISCUSSION

In the present work employing the accuracy of 50% hemolysin units determined photometrically, a total of 16 doses of 400 r hard X-rays given to 9 rabbits during the decline of hemolysin failed to yield a single unequivocal anamnestic rise in antibody. One animal exhibited a change from a rapid decline of hemolysin to almost a plateau about the time of the first treatment (rabbit 3, fig. 1). This sequence, however, cannot be ascribed to irradiation because a similar slowing in rate of antibody decline generally takes place at about this time (cf. the other rabbits in fig. 1 and our previous work, 1950).

Our previous studies indicated that following a single injection of sheep cells in the quantities used in experiment 1, the rise and decline of hemolysin in rabbits fit a series of discontinuous curves described by a monomolecular reaction equation in which the constant of increment varies. The constant of increment during the initial rise was usually between 1 and 2, and

the decline from the peak indicated a half life of 5.5 ± 0.3 days for serum antibody. Although peak titers were highly variable, the general nature of the various curves were remarkably uniform in shape. Data on the rabbits in this study indicate that X-irradiation did not actually modify the normal antibody curve. In figures 1 and 2 of this paper, the data are not fitted to theoretical curves as in the first paper because determinations were not as closely spaced and the multiply injected animals show an irregular plateau while receiving antigen.

The question might be raised as to whether or not serum from our animals was titrated soon enough after irradiation. We bled the rabbits in experiment 1 one day after irradiation and the ones in experiment 2 as soon as Dougherty and White (1946) bled their intact mice receiving 200 r, i.e., at 6 hours. We believe even the 24-hour bleeding is adequate, however, since any considerable amount of antibody, had it been released into the serum, would be apparent after 1 day unless its half life was shorter than that observed by us. A possible exception may be the temporary slight reaction two days after irradiation in rabbit 13 (fig. 2), but seems too delayed to us. It is probably an aberrant reading due to a technical error for it is similar to the irregular rise in control rabbit 5 (fig. 1), on the 23rd day after immunization.

Our negative results cannot be explained by the lack of lymphocytocidal activity of the X-rays. In the first place, treated rabbits showed a marked lymphopenia. In the second place, the source of radiation, experimental conditions of irradiation and the source of rabbits were identical to those used by Murray (1948) and De Bruyn (1948) to obtain material for the descriptions of damage, after single doses of 400 r, to the spleen and to the lymph nodes and intestinal lymphatic tissues, respectively.

The present negative results, on the one hand, are contrary to the work of White and Dougherty (1945) and Dougherty and White (1946), who produced a nonspecific anamnestic rise in antisheep agglutinins in mice with 200 r X-rays administered after the titer was not titratable in intact mice and after it had reached the comparatively low figure of 1:80 in adrenalectomized mice. On the other hand, they are in agreement with the failure of Craddock and Lawrence (1948), using 250 r, and Fischel et al (1949), using 50 r, to produce a rise in antibody titer in rabbits. They specifically corroborate Craddock and Lawrence's results with rabbit antisheep hemolysins and extend them in that they involve a higher dose of X-rays and eliminate the possibility that Craddock and Lawrence's results with 100% hemolytic units may have missed small differences.

It should also be pointed out that investigators early recognized the importance of the time at which X-rays should be administered to affect antibody production. Thus, large doses at about the time of antigen injection reduce immunity, whereas when postponed even for several days they are generally ineffective. Most of the reports of enhanced antibody formation following irradiation at appreciable intervals after immunization involve small doses frequently applied locally (review by us, 1951).

Using another lymphocytocidal agent, Philips et al (1947) failed to produce nonspecific anamnesis with daily leucopenic doses of tris(beta-chloroethyl)-amine in goats which had been previously immunized with ricin and their

serum antibodies allowed to fall to negative levels.

Our results are also contrary to the frequently held hypothesis of nonspecific anamnesis induced by unrelated antigens (review in Cannon, 1942), whether induced directly or indirectly through pituitary-adrenal cortical control. We failed to induce such an anamnestic rise in hemolysin with ovalbumin recrystallized three times. There was probably an anamnestic rise in one animal after one injection of the crystalline product and two injections of a crude product. Both preparations elicited a marked homologous precipitin response. Our results, however, are in accord with several studies using quantitive precipitin methods (see Heidelberger and Kendall, 1935, Kabat and Heidelberger, 1937, and van der Scheer, Bohnel, Clarke and Wychoff, 1942). In unpublished work we have also found that when rabbits previously immunized with sheep cells are injected with duck cells, the antisheep hemolysin rises about 20%. The absolute value of these rises could probably not have been detected with 100% end points.

The present results agree with other recent failures to demonstrate antibodies in lymphoid cells or lymphoid tissue early in the process of immunization such as described by Dougherty et al (1944) and Harris et al (1945). Thus, Craddock et al (1949), in their study of H and O typhoid agglutinins in the cat, were unable to obtain evidence for the release of antibody by exposures of 1500 r or for the presence of antibody within lymphocytes during the induction period and early antibody rise when it would be expected that antibody formation is at its height. In addition, possible technical objections associated with carrying over antibody on the cells and the walls of test tubes have been raised against recent demonstrations of antibodies in lymphocytes (cf. Spear, 1948, and Habel et al, 1949). Somewhat similarly, in our recent study (1950) on hemolysins, we have shown that splenectomy reduces the initial rise of antisheep hemolysins, on the average, by 80% and have presented evidence that the spleen is remarkably active for several days and then abruptly ceases to form antibody. In spite of this evidence of high activity, we never found a higher hemolysin titer in the spleen than in the serum except at times during the induction period when serum titers were low and antibody titrations were comparatively unreliable.

In spite of these failures both by others and ourselves to find antibody in lymphocytes, we believe there is overwhelming evidence of the importance of lymphoid cells (including lymphocytes, various "blasts" and the large basophil lymphoid cells which have been given various designations including developing or young plasma cell) in immunity and highly suggestive evidence that they, at least in part, are involved in antibody formation and/or transport (review by the senior author, 1949). For the present, we prefer to consider that certain fixed and free cells in lymphoid tissue are involved in antibody formation but that the antibody is released from the cell almost as soon as it is recognizable as antibody. We also recognize the fact that various biological and technical factors may explain part of the discrepancies in published work, as stressed by T. N. and S. Harris (1950).

CONCLUSIONS

X-irradiation in 2 doses of 400 r to the entire body of rabbits during the decline of hemolysin 2 to 4 weeks after immunization with one or a series of intravenous injections of sheep red cells did not alter the downward trend of

the titer as compared to controls not irradiated.

Three intravenous injections of ovalbumin 9½, 11 and 12 weeks after immunization only elicited a possible heterologous anamnestic response to red cells in 1 of 7 rabbits.

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TRANSMISSION OF THE HAMSTER-ADAPTED NEWCASTLE VIRUS TO THE *MACACUS RHESUS* MONKEY

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Following the adaptation of Newcastle disease virus, California strain No. 11,914, to the Syrian hamster,[1] attempts to transmit the modified virus of the 21st hamster passage to the *Macacus rhesus* monkey were successful through the first subinoculation only.[2] Further attempts to establish the infection in *M. rhesus* monkeys by the injection of later hamster passages were likewise unsuccessful beyond the first subinoculation until the present study. Wenner and his associates reported the adaptation of two strains of NDV to rhesus monkeys and the subsequent brain-to-brain passage of this virus for three serial passages.[3]

A virus suspension from the 310th hamster passage was prepared from 10 infected hamster brains. The brain tissue was ground with alundum and diluted to a 10% suspension with physiological saline solution. This suspension was centrifuged in an angle centrifuge for 5 minutes at 1000 r.p.m. to rid the suspension of heavy tissue particles. Approximately 3 cc of this 10% virus suspension, which titered $10^{-4.5}$ intracerebrally in hamsters by the Reed and Muench method of calculation,[4] was injected intracerebrally into each of 2 *M. rhesus* monkeys (weight 7 lbs.). A 1-inch 21-gauge needle was used for inoculation. The inoculation was made 1 cm to the right of the midline and 1 cm anterior to the coronal suture, and the 3 cc of 10% virus suspension was inoculated into the right frontal lobe. The monkeys were not anesthetized before inoculation. The use of this Newcastle virus of the 310th hamster passage for the initial monkey inoculation established an infection in which 50% or more of the injected monkeys of the first 6 passages showed symptoms of central nervous system involvement. From the 7th to the 10th passage, 100% of the injected monkeys showed similar symptoms. Table 1 shows the results of these 10 serial passages. These monkey passages are being continued at the present time.

TABLE 1.—*Monkey passage of a strain of hamster-adapted Newcastle virus*

Passage no.	*M. rhesus* monkeys inoculated	Monkeys showing symptoms*	Days after inoculation symptoms appeared
1	2	2	4, 9
2	3	2	8, 10
3	3	2	4, 5
4	4	2	4, 6
5	4	3	4, 5, 7
6	4	3	4, 5, 8
7	2	2	5, 6
8	2	2	4, 5
9	3	3	3, 3, 3
10	2	2	3, 4

* These symptoms included irritability and malaise, involuntary motor reactions, paralysis, excessive salivation, and prostration.

In monkeys responding to the virus injection, irritability and malaise ap-

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peared in 3 to 10 days. In most cases these symptoms were followed by rhythmical contractions of muscles starting in the limbs and spreading over the entire body, paralysis of extremities, excessive salivation from pharyngeal paralysis, prostration, and death.

Brain material for passage was chosen from monkeys sacrificed when nervous symptoms developed. The virus-bearing brain material was chopped with scissors and ground with alundum in a large mortar and diluted to a 10% suspension with physiological saline solution. Amounts of 3 cc of 10% suspension were injected intracerebrally throughout the 10 passages.

Infected monkey brain suspensions of the 1st to the 10th monkey passage, prepared as previously described, were injected intracerebrally into 4 young Syrian hamsters (age 21 days). Within 24 to 72 hours after inoculation, all hamsters showed symptoms characteristically seen in animals succumbing to similar injections of hamster-adapted virus.

Two-tenths cc of brain suspensions from the 2nd monkey passage was injected into the allantoic sac of 9-day embryonated chicken eggs. Only 8.3% of the chick embryos died upon this 1st passage. One hundred percent of the embryos died upon the 2nd passage, and the allantoic fluid harvested from these infected eggs was used in neutralization tests conducted in 9-day embryonated chicken eggs. Newcastle immune chicken serums completely neutralized the virus while normal chicken serums had no effect.

Attempts to isolate the virus in embryonated eggs directly from monkey brains were unusccessful with the 4th through the 8th monkey passages. This would seem to indicate that the pathogenicity of the virus for chicken embryos has been reduced by passage of the virus through monkeys. However, virus was isolated in 9-day embryonated eggs inoculated with 0.2 cc of a 10% brain suspension from hamsters infected with virus from the 8th monkey passage. A neutralization test in 9-day embryonated eggs, using the allantoic fluid from these infected eggs, was positive for Newcastle disease virus.

Two virus neutralization tests were conducted, using brain suspension of the 8th monkey passage with normal and specific Newcastle immune chicken serums. In the 1st neutralization test rhesus monkeys were used as the test animals; in the 2nd test, 21-day-old Syrian hamsters were used. In both tests specific immune serums completely neutralized the monkey brain virus, whereas normal chicken serums had no effect. The virus from the 8th monkey passage titered $10^{-2.5}$ in hamsters by intracerebral inoculation according to the Reed and Muench method of calculation.[3]

In all neutralization tests the monkey brain virus was neutralized by specific Newcastle immune chicken serums and was unaffected by normal chicken serums. The possibility of the presence of another virus as the cause of the disease shown in monkeys and hamsters seems remote.

SUMMARY

The hamster-adapted Newcastle virus (California strain No. 11,914) of the 310th passage has been successfully transmitted to the *Macacus rhesus* monkey and carried through 10 serial passages in this species by intracerebral inoculation. The virus from the 8th monkey passage titered $10^{-2.5}$ in hamsters injected intracerebrally. The virus produced symptoms of irritability and malaise usually followed by a characteristic nervous jerking of all limbs, salivation caused by pharyngeal paral-

ysis, paralysis of extremities, and death. Hamsters showed similar symptoms following injection of the virus-bearing monkey brain suspensions.

Virus isolated in embryonated eggs from the 2nd intracerebral monkey passage and from hamsters infected with the 8th monkey passage proved to be Newcastle disease virus by neutralization tests in embryonated eggs. Neutralization tests conducted in monkeys and hamsters, using the 8th monkey passage brain suspension, also verified the virus to be that of Newcastle disease.

ELECTRON MICROGRAPHS OF NEGRI BODIES FOUND IN RABIES

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Specific inclusion bodies present in the central nervous system of man and animals dying of rabies are called "Negri bodies."[1,2] These are most frequently demonstrated within, but occasionally outside of, the nerve cells. A definite diagnosis of rabies is made upon demonstration of these bodies in the nerve cells of the brain in stained smears or cut stained tissue sections.

These inclusions are eosinophilic staining bodies, usually spherical but also of other shapes, varying from 1 to 30μ^3 in size. The inner structure shows basophilic staining granules.

Negri, upon discovery of this body, believed it to be a protozoan. According to one theory the Negri body may be composed of elementary bodies, but there has been no entirely satisfactory evidence to indicate that the body is composed of virus particles.

In the present study electron micrograph examinations were attempted with histological sections of brain tissue containing Negri bodies, but these proved to be unsatisfactory because the electron beam would not penetrate the specimen section used. Chemical digestion of the brain cells by trypsin was attempted, but the inclusion bodies seemed to be disintegrated in this process. The method to be reported herein seems to work satisfactorily and brings out, as illustrated, some details of structure.

MATERIAL AND METHODS

The viruses for the present study were obtained from dog brains sent to this laboratory for routine examination for rabies. The hippocampus was removed from each brain aseptically, ground with alundum in a mortar, and diluted with physiological saline to a 10% suspension. Three hundredths ml of this 10% suspension was injected intracerebrally into each of 6 4-week-old Swiss albino mice (Carworth Farm Webster strain). On the 14th day after injection of the first strain several mice showed tremors and paralysis and became moribund. The brains were removed aseptically from these moribund mice. Impressions made from the Ammon's horn of each mouse were stained with Sellers stain[4] and under the light microscope showed numerous Negri bodies throughout the tissue. The Ammon's horn from one mouse contained a greater number than the others and this was used for the experiment below.

The second strain of rabies virus was injected intracerebrally into 6 Swiss mice and on the 7th day after injection, several mice showed tremors and paralysis and became moribund. The Ammon's horn containing the greatest number of Negri bodies was used for the electron examination.

Parlodion film supports were prepared 24 hours before being used. The prepared screen was picked up with forceps and the parlodion surface was dipped in 10% formalin. The parlodion surface was then touched lightly to the same area of the Ammon's horn used for direct smear examination. These screens were left to dry for examination the following day.

Several screens from each strain were examined in an EMU electron microscope at various magnifications for neurons and inclusion bodies. The electron micrograph (see fig. 1) shows a nerve cell with an inclusion body located on the inner surface. No internal structure is observable. It was noted throughout this study that internal

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structure could not be demonstrated in all inclusion bodies, probably due to the combination of cell wall and body thickness, or the latter alone, which blocked out electron penetration. The electron beam from a Bias gun was tried but this beam did not bring out the structure of the Negri body in any more detail, despite the increase in intensity of electrons.

By proper adjustment of the exact exposure time in photographic technique the inclusion body in figure 2 shows an internal structure. Several photographs of the same inclusion body at various exposure times are presented.

In figure 3 two extracellular Negri bodies are shown. One of these shows internal structure and the other does not. Figure 4 is used for comparison. It is a photomicrograph of a stained (eosin-methylene blue) tissue section from a mouse brain. Figure 5 shows a Negri body from the second strain. It shows definite virus-like particles similar to those seen in the examination of the first strain. Figure 6 shows an inclusion body partly covered by a nerve cell. This body shows definite round virus-like particles.

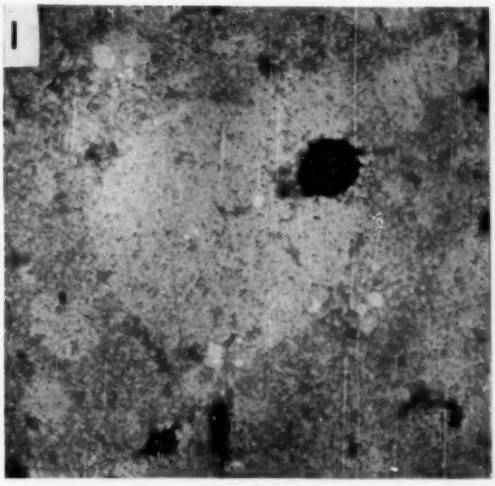
DISCUSSION

Electron micrographs of parlodion-prepared screens show that it is possible for electrons to penetrate some of the inclusion bodies. An indefinite internal structure is demonstrated in both strains examined. The Bias gun was tried but did not bring out any more detail than the ordinary EMU electron gun. In figures 1, 2, 3, 5, and 6 preparations were examined unstained. Figure 4, a photomicrograph of a stained preparation, was used for comparison with the electron micrographs.

SUMMARY

Negri bodies in this electron microscope study seem to have indefinite internal structure which might be in the form of elementary bodies. It was not possible to bring out more detail, probably due in some cases to the combined thickness of the nerve cell and the density of the Negri body, which partly blocked out electron penetration.

This technique of specimen preparation for electron studies seems to be satisfactory on examination of these two rabies strains.



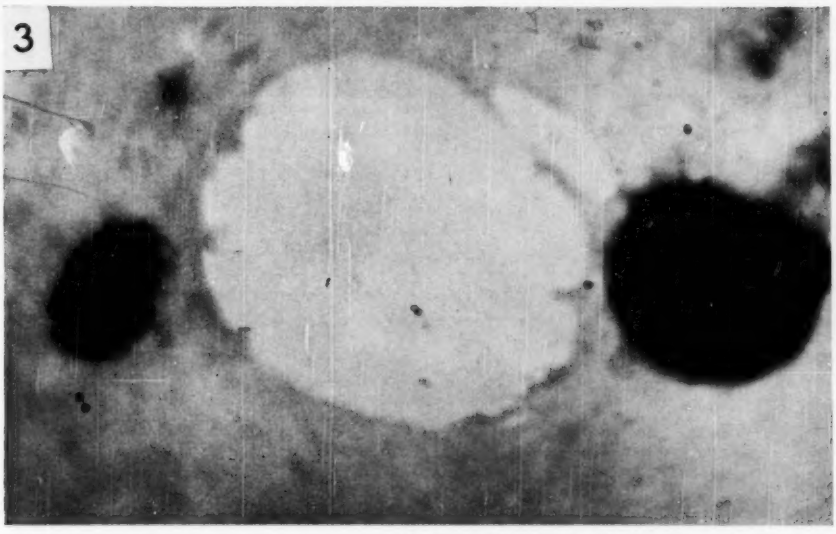
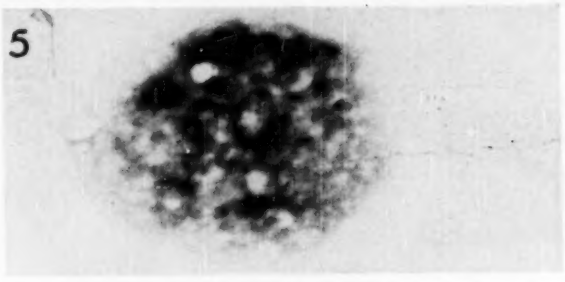


FIG. 1.—Electron micrograph of a mouse brain cell containing a Negri body. Internal structure not evident. ×9100.

FIG. 2.—Electron micrograph of a Negri body showing virus-like particles brought out by adjustment of the exposure time. ×27,300.

FIG. 3.—Electron micrograph showing two Negri bodies, one of which shows an internal structure. × 18 200.



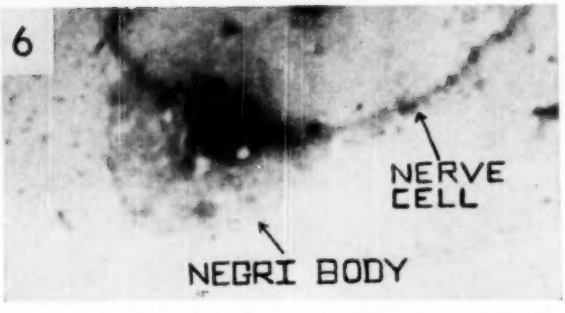


FIG. 4.—Photomicrograph of stained (eosin-methylene blue) tissue preparation from Ammon's horn of a mouse showing Negri bodies with slight indication of internal structure. ×3000.

FIG. 5.—Electron micrograph of a Negri body in the edge of a nerve cell of a mouse. ×30,000.

FIG. 6.—Electron micrograph of a Negri body from the second strain showing round virus-like particles. ×20,000.

STUDIES OF FUSOSPIROCHETAL INFECTION

I. PATHOGENICITY FOR GUINEA PIGS OF INDIVIDUAL AND COMBINED CULTURES OF SPIROCHETES AND OTHER ANAEROBIC BACTERIA DERIVED FROM THE HUMAN MOUTH

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The production in rabbits of foul gangrenous abscesses by subcutaneous inoculation of material from the human mouth or from lung abscess was first reported by Veszpremi[1] and has been confirmed repeatedly (see Rosebury and Foley[2]). Guinea pigs have been most commonly employed, and in a recent comparative study[3] this species was found more suitable for the purpose than other common laboratory animals. In the guinea pig the experimental infection has been initiated with scrapings or exudates from any of several common oral diseases,[4] from Vincent's angina[5] or from pulmonary abscess or gangrene.[6] It is readily passed from animal to animal by subcutaneous inoculation and has consistent pathological features,[3] including necrosis and a fibrinopurulent exudate, characteristically abundant and foul and containing large numbers of anaerobic microorganisms, including spirochetes, fusiform bacilli, motile vibrio-like forms, streptococci and other bacteria. While there is general agreement in the literature on these findings, there have been few attempts to analyze such exudates bacteriologically to determine the part played by the component microorganisms in the etiology of the process. It is generally agreed that no single species in the mixture can itself be held responsible for the lesions, since with few and apparently unimportant exceptions individual pure cultures of them have been found nonpathogenic for laboratory animals. Veszpremi[7] and Paul[8] reported that typical lesions could be produced in rabbits and guinea pigs, respectively, with unpurified culture mixtures containing spirochetes, fusiform bacilli and cocci. Kirtchewski and Seguin[9] also recorded successful results by subcutaneous inoculation of guinea pigs with mixtures containing any of three cultures of spirochetes with fusiform bacilli and either a greening streptococcus ("strepto-viridis") or a white staphylococcus. The latter workers stated that combinations of fusiform bacilli with either coccus were ineffective, but that "the addition of a trace of spirochetes" to such mixtures restored their pathogenicity and reproduced the characteristic abscess. Few experimental details were given. It is recorded that a mixed culture of fusiform bacilli and *Spirocheta*

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acuta, grown together in ascitic fluid broth containing rabbit kidney, yielded lesions in guinea pigs indistinguishable from those initiated with gingival scrapings. *S. acuta* was thought to correspond with Noguchi's[10] *Treponema macrodentium;* two other spirochetes (*Spirocheta dentium*, syn. *Treponemc microdentium*, and *Spirocheta tenuis*, an intermediate form) appear to have been tested only in the presence of cocci.

In 1930, Smith[5] reported the isolation from guinea pig lesions, initiated with material from Vincent's angina, of *Staphylococcus albus* and *Streptococcus viridans* on blood plates incubated aerobically, and by several anaerobic techniques, of hemolytic streptococci, a gram-negative motile vibrio, two fusiform bacilli (a small nonmotile form and a larger form, thought to be motile originally but nonmotile in culture), and *Tr. microdentium*. The vibrio and the anaerobic streptococcus were described as producing fatal infections in animals in pure cultures if inoculated in large doses. Mixtures of the spirochete with "both the large and the small fusiform bacilli failed to produce any infection in either mice or guinea pigs." The streptococcus and the vibrio, when mixed, yielded a severe infection, which, however, did not resemble the original fusospirochetal abscess. When the small fusiform bacillus was added to this mixture, "a small abscess was produced which contained an odorless green pus." When the spirochete was added to these three organisms, however, the mixture was said to yield a typical fusospirochetal lesion "which could be transmitted from mouse to mouse, and from mice to guinea pigs, by injecting as small amounts as 0.25 cc. into the groin." In commenting on this finding later, Smith[11] considered it "very likely that any of the oral spirochetes, several of the fusiform bacilli, and several of the cocci could replace the ones actually used in this experiment." In this reference Smith described as occurring in fusospirochetal material a wider range of microorganisms than were included in his recombination experiment, but he seems not to have attempted to determine whether any but the indicated four or five could be implicated in fusospirochetal infection.

This deficiency was made up in part by the studies of Proske and Sayers,[12] in which the findings of Smith were recorded as having been duplicated with remarkable consistency and some amplification. In a study of pulmonary fusospirochetal infection secondary to silicosis, these workers also made cultures of the flora of inoculated guinea pigs, and presented useful details of their cultural methods. They reported isolating the following anaerobes by these means: *Tr. microdentium*, *Treponema mucosum*, *Tr. macrodentium*, *Treponema buccale*, a "culture of fusiform bacilli in which spirochetes [*T. vincenti*] had developed," two types of *Bacillus fusiformis*, *Spirillum sputigenum*, *Vibrio viridans*, and a nonhemolytic streptococcus which, like the vibrio, lost its strictly anaerobic habit after being subcultured. These organisms were described systematically. It was not indicated whether more than a single culture of each was studied nor, indeed, which of the given characteristics of each culture were observed by the authors and which were taken from the literature. The nine pure cultures

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11. Smith, D. T. 1932, Oral Spirochetes and Related Organisms in Fuso-spirochetal Disease, Baltimore, Williams & Wilkins Co.

12. Proske, H. O. and Sayers, R. R. 1934, Pub. Health Rep. **49**: 839, 1212.

were prepared as heavy suspensions in 0.85% sodium chloride and standardized against human erythrocytes to contain about 10^8 organisms per ml. The mixed culture could not be thus standardized. Pathogenicity tests with individual cultures showed the spirochetes, fusiform bacilli and spirillum to be nonpathogenic. Either the streptococcus or the vibrio produced mild lesions in small doses, while in large doses each "occasionally caused death evidently from toxemia, because no pathological changes were noted upon autopsy." Combinations of either the streptococcus or the vibrio with each of the other cultures in turn yielded slightly more promising lesions in guinea pigs with *T. microdentium*, *T. mucosum* and the two fusiform bacilli than with the other cultures. Combinations were then tried with both streptococcus and vibrio, which together produced a much more severe lesion than was obtained with either alone. These three-organism combinations again gave most promising results when they contained the small spirochetes or the fusiform bacilli than when *T. macrodentium*, *T. buccale*, the fusiform-vincenti mixture or *S. sputigenum* were present. None of these lesions resembled a typical fusospirochetal abscess. Nine combinations were then made from the 6 cultures that seemed most effective, with all 6 cultures and combinations of 5 and 4, all including streptococcus, vibrio and at least 1 spirochete and 1 fusiform bacillus. All these combinations were said to yield a "typical abscess with necrosis, greenish-gray foul-smelling pus." Moreover, "all possible combinations of the 10 types of organisms" were studied, but only combinations that included the streptococcus, the vibrio, either of the tightly wound spirochetes and either of the fusiform bacilli yielded typical fusospirochetal lesions. Proske and Sayers state further that "the same reactions were obtained when as few as 10 million organisms of each type were used in the combinations. The lesions can be transferred from guinea pig to guinea pig, but after a few passages the organisms become so virulent that the animals die from toxemia before a lesion can develop."

The findings of Smith and of Proske and Sayers are reviewed in detail because they formed the basis for initial studies in this laboratory. The original plan of this work was to utilize a fusospirochetal infective mixture of pure cultures for studies of the mechanism of predisposition that seems to be required in naturally occurring infections of this type. It became apparent, however, that the problem is more complex than would appear from the published record. Not only are the individual organisms named in the literature inadequately identified, but the range of types given by no means includes all the forms recoverable both from human oral lesions and from well established guinea pig passage exudates. The results of the present studies seem to warrant a revaluation of this problem. This report presents data on two series of experiments in which the cultures used were derived from several human sources, in most instances directly from gingival scrapings and in some from animal exudates after passage. In other reports the results are given for (a) an attempted analysis and quantitative recombination of the organisms in a single guinea pig passage strain of fusospirochetal infection,[13] and (b) recombination experiments based on the findings in repeated mixed subcultures of

13. Rosebury, T., Clark, A. R., Tergis, F. and Engel, S. G. 1950, J. Infect. Dis. **87**: 226–233.

whole exudate from the same passage strain.[14]

METHODS AND MATERIALS

The cultures employed were obtained in the course of routine anaerobic cultivation of gingival scrapings from unselected cases of gingival and peridontal disease.[15] The 15 strains used were derived from 11 cases, mainly by direct culture of gingival scrapings, in 3 instances after guinea pig passage, and in 1 of the latter after an additional passage through mouse brain.[8] The procedure for primary cultivation consisted of inoculation of at least two "AFK" spirochete plates,[16] serial streaking on 2 or more blood agar plates, and heavy inoculation of small plates (17×60 mm over-all) of gentian violet agar. These and other mediums, which were used less regularly, are described below. Primary cultures were incubated at 37 C for 2 to 8 days in sealed jars made anaerobic with hydrogen catalyzed with electrically heated platinized asbestos. Anaerobiosis was checked by reduction of methylene blue in 2% dextrose-phosphate buffer, pH 8.3. For identification of *Fusobacterium* the morphological criteria of Bøe[17] were followed. For the other types of organisms, criteria in the literature for the identification of potential pathogens seemed too imperfect to justify systematic study of each culture until after its pathogenicity had been established. Previous workers have been in general agreement that a spirochete of the *microdentium-mucosum* type is concerned, that the requirement for a fusiform bacillus is not precise, and that one or more ill-defined streptococci may be effective. Since in the writers' experience no anaerobic vibrios showing the greening on blood agar described by Smith[8,11] and Proske and Sayers[12] were met with, 4 strains with different characteristics were selected. In addition, 3 strains of presumed *Bacteroides* and 1 of *Leptotrichia* were used, the former because of their reported pathogenicity in pure culture,[18–20] the latter because it has apparently been mistaken for a large fusiform bacillus.[17,21] In the hope of avoiding possible changes in pathogenicity resulting from prolonged artificial cultivation during the period of collection of these cultures, each one was provisionally identified by morphological means when isolated, tested for aerobic growth, and the earliest available pure subculture stored until needed by freezing in solid carbon dioxide. The effectiveness of this method in maintaining viability of cultures of the oral anaerobes and in preserving the infectivity of fusospirochetal exudates is reported on elsewhere.[22]

Mediums.—The composition of the AFK and SUK mediums, used principally for isolation and maintenance of spirochetes, is given in another report.[16] Both were made with the Proske-Sayers[12] "hormone" base and contained 1.2% agar, 30% ascitic fluid (AF) or serum ultrafiltrate (SU), 0.1% cysteine hydrochloride and minced fresh guinea pig kidney. Other mediums used were: blood agar—heart infusion, 2% agar, pH 7.6, with 10% defibrinated sheep blood; brain-heart agar—Bacto brain-heart infusion (No. B37) with 2% agar; enriched veal infusion agar—Bacto veal infusion medium with 2% agar added, to which was added aseptically ascitic fluid, 30%, dextrose, 0.2%, and neutralized cysteine hydrochloride, 0.1%, final pH 7.3–7.6; gentian violet agar—enriched veal infusion agar as above with 0.003% gentian violet added; enriched veal infusion broth—Bacto veal infusion medium (containing 0.1% agar), to which was added aseptically ascitic fluid, 10%, dextrose, 0.1%, and neutralized cysteine hydrochloride, 0.1%, final pH 7.3–7.6; thioglycollate medium—Bacto Brewer medium No. B,36; Robinson-Wichelhausen broth[23]—the 1% rabbit blood, agar-free medium, slightly modified from that of the original authors, as described elsewhere.[16]

Distilled water solutions of dextrose (20%), cysteine hydrochloride (10%) and gentian violet (0.1%), as well as normal sodium hydroxide used for titration, were sterilized by autoclaving at 15 pounds pressure for 20 minutes and added

14. Rosebury, T., Clark, A. R., Macdonald, J. B. and O'Connell, D. C. 1950, J. Infect. Dis. **87:** 234–248.
15. Rosebury, T., Macdonald, J. B., Clark, A. R. and Frances, S. J. Dent. Research. In press.
16. Rosebury, T., Macdonald, J. B., Ellison, S. A. and Engel, S. G. Oral Surg., Oral Med. & Oral Path. In press.
17. Bøe, J. 1941, Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No. 9.
18. Guillemot, L., Halle, J. and Rist, E. 1904, Arch. Med. exp. et d'Anat. path. **16:** 571.
19. Beaver, D. C., Henthorne, J. C. and Macy J. M. 1934, Arch. Path. **17:** 493.
20. Smith, W. E. and Ropes, M. W. 1945, New England J. Med. **232:** 31.
21. Thjøtta, T., Hartmann, O. and Bøe, J. 1939, Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No. 5.
22. Rosebury, T. and Frances, S. Oral Surg., Oral Med. & Oral Path. In press.
23. Robinson, L. B. and Wichelhausen, R. H. 1946, Bull. Johns Hopkins Hosp. **79:** 436.

to the mediums aseptically. All mediums were tested for sterility by incubation at 37 C aerobically for 24 hours, then anaerobically in hydrogen jars for 48 hours. Except for thioglycollate medium, which was heated in a water bath at 100 C and cooled rapidly before inoculation, all mediums were stored in anaerobic jars at room temperature until needed. All cultures including those in thioglycollate medium, but excepting cultures of spirochetes in the AFK medium in Noguchi tubes, were incubated anaerobically.

The following cultures were used in these studies:

Spirochetes.—TM8. From gingival scrapings, case BB (Vincent's gingivitis). AFK plate, 7 days anaerobically at 37 C, from a diffuse haze.[16] A consistently tightly wound spirochete typical of *T. microdentium.*[10]

TM11. From gingival scrapings, case GP (Vincent's gingivitis). SUK plate, 6 days anaerobically at 37 C, from a demarked haze.[16] A variable tightly wound spirochete.

Except as otherwise noted the following strains were isolated directly from gingival scrapings in cultures incubated anaerobically at 37 C, failed to grow aerobically on blood agar and grew in thioglycollate broth.

Fusobacterium.—F12. From case JG (periodontoclasia); isolated from a brain-heart agar streak culture incubated 5 days. Morphology typical of *Fusobacterium* as described by Bøe.[17]

F28. From case GM (periodontal abscess), on gentian violet agar streak, 5 days. Typical *Fusobacterium.*

Vibrios.—VB2. From case JM (marginal gingivitis); separated, probably incompletely, from spirochetes in an AFK plate, after 4 transfers through AFK medium in Noguchi tubes. Small vibrios and larger spirilla-like forms, gram-negative, active darting and tumbling motility. No growth on blood agar anaerobically. Light growth in enriched veal infusion broth, not in thioglycollate medium.

VB4. From case ML (marginal gingivitis), on blood agar streak, 8 days. Colony 1 mm, flat umbonate, opaque center, petalled edge. Small comma-shaped and spirillar forms, variably gram-negative, actively motile.

VB5. From case MW (periodontoclasia). Isolated, from mixed culture on an 8-day blood agar streak culture, after 4 subcultures in thioglycollate broth and 1 on blood agar anaerobically. Colony after 5 days less than 1 mm, transparent, umbonate. Slender, straight, curved and double-curved gram-negative rods, motile, flagellated.

VB6. From case GE (periodontoclasia). Isolated, from mixed culture with *Leptotrichia*, in a SUK spirochete plate, by streaking on blood agar anaerobically. Colony after 4 days, up to 1 mm, graying, low cone-shaped, smooth matt confluent. Small, straight, curved and double-curved rods, some chains, some filaments. Gram-negative, actively motile.

Streptococci.—S3. Case IS (marginal gingivitis), on blood agar streak, 8 days. Colony more than 1 mm, rough, convex, translucent. No action on blood. Large gram-positive coccus in long chains.

S17. From 4th passage guinea pig exudate from case IS (marginal gingivitis), on enriched veal infusion agar streak, 3 days. Colony less than 0.5 mm, low, convex, translucent, pale grayish, smooth. Variable gram-positive cocci in pairs and short chains.

S20. From 7th passage guinea pig exudate from case AW (periodontoclasia), on blood agar streak, 2 days. Colony, more than 1 mm, smooth, opaque. No action on blood. Medium gram-positive cocci in pairs, short and long chains.

Anaerobic gram-negative rods (presumptively *Bacteroides*).—B6. From case MW (periodontoclasia), on blood agar streak, 4 days. Colony less than 1 mm, low, convex, smooth, yellowish center, translucent edge. Delicate gram-negative rods, nonmotile. Better growth in serum broth and in enriched veal infusion broth than in thioglycollate medium.

B13. From case MB (periodontoclasia). Separated from spirochetes in a 7-day AFK spirochete plate by growth in enriched veal infusion broth. Colonies on blood agar at 4 days, less than 1 mm, gray, translucent smooth, with minute rough depressed variants. Both types showed delicate granular gram-negative rods and filaments, nonmotile, with some fusiform and some funduliform elements, and some forms showing single contours under darkfield illumination.

F26. From 12th passage guinea pig exudate followed by mouse brain inoculation,[3] from case AW (periodontoclasia). Isolated from a gentian violet agar plate.

Leptotrichia. L1. From case MW (periodontoclasia), in an AFK spirochete plate, 5 days, from a fibrous haze,[16] on blood agar streak, 4 days. Colonies less than 1 mm, translucent, "medusa-head" or rhizoid. Coarse gram-positive granular rods and filaments, some with one or both ends pointed; easily decolorized by alcohol; nonmotile. Growth in enriched veal infusion broth.

Procedure for pathogenicity tests.—Mass cultures were prepared in 125 ml Florence flasks filled with fluid medium to the neck. For the two spirochete strains, Robinson-Wichelhausen broth was used; for all others, enriched veal infusion

broth. For the first experiment the flasks, after having been incubated anaerobically for 48 hours, were inoculated directly from freshly thawed seed cultures, except for strain TM11, for which a recently isolated AFK culture was used. In this series the flask culture of strain TM8 was found to be contaminated, and a culture in an AFK tube was substituted, approximately 2 ml of spirochete-laden agar from this culture being emulsified in 5 ml of suspending medium consisting of 0.1% gelatin and 0.9% sodium chloride in distilled water. This suspension contained approximately 10 spirochetes per oil-immersion field, as compared with the final suspension of strain TM11 in flask culture, which showed approximately 200 spirochetes per field. The flask cultures, after removal of about 20 ml from the upper portion and shaking with sterile glass beads, were centrifuged at approximately 6000 r.p.m. for 45 minutes. The sediment, consisting (except for TM11, which contained no agar) of about 5 ml of agar gel in each of two 50-ml centrifuge tubes, was resuspended and pooled for each strain by addition of 10 ml of gelatin-saline solution. Attempts to standardize these suspensions with the aid of a Klett-Summerson photoelectric colorimeter were unsuccessful, apparently because of the inhomogeneity of the agar-gel suspensions. Microscopic counts against human red blood cells stained by Wright's method indicated that the suspensions contained from 0.45 to 2×10^9 organisms per ml. Satisfactory counts of the spirochete suspensions could not be obtained by this method.

For the second experiment additional strains were selected in an effort to provide cultures isolated from guinea pig passage exudate; but 3 of these (2 spirochete strains that failed to grow in Robinson-Wichelhausen broth, and a strain of *Fusobacterium*, the initial culture of which was broken) proved unusable. The group, however, included 2 such strains (S17 and S20) and 2 strains (F12 and B13) which had been reisolated from pure culture lesions in the first experiment, as noted below. In an effort to enhance growth of all cultures used in this experiment, the freshly thawed seed cultures were first grown for 48 hours, the spirochetes in Robinson-Wichelhausen broth, the other cultures in enriched veal infusion broth, and then inoculated as before into flasks of the same mediums for the respective groups. This procedure seemed effective for all cultures except spirochetes, since counts against human erythrocytes of suspensions prepared as in the first experiment were now found to range from 1.2 to 4.9×10^9 organisms per ml. None of the spirochete strains used, however, grew in the flask cultures. A suspension was therefore prepared by pooling the spirochete-laden agar from 3 AFK tube cultures of TM11 in suspension with 10 ml of gelatin-saline solution. This suspension was found to contain approximately 25 spirochetes per oil-immersion field.

Inoculums for guinea pigs were prepared throughout by mixing equal volumes of cultures, except for strain TM8 in the first experiment. Since this strain was in short supply it was used in the proportion of 0.5–0.7 ml to 1 ml of other strains. All guinea pigs in both experiments received 1 ml subcutaneously in the shaved inner aspect of the upper thigh, followed by traumatization of the injected area with the needle. As indicated in the tables, the 11 strains used in the first experiment were inoculated individually, in selected groups of 4, 5 and 6 cultures, and in a pool containing all 11. In the second experiment the 10 strains used were inoculated individually and in selected groups of 2, 3 and 4 strains. Two guinea pigs were used in each group except for the groups of 6 and 11 cultures in the first experiment, in which 3 animals were used, and the individual inoculation of strain TM11 in the second experiment, in which only enough material for a single animal was available.

The guinea pigs were examined daily for 8 to 14 days, when they were killed and autopsied. In animals showing evidence of fluctuating abscesses, aspiration of exudate was attempted as early as the 6th day after inoculation. When exudate could be obtained it was examined under the darkfield microscope and, if the findings warranted, passed to a fresh guinea pig. In several instances in the first experiment only, exudate from animals inoculated with individual cultures were streaked on sheep blood agar and incubated anaerobically for reisolation of the inoculated organism.

RESULTS

The data in table 1 indicate that in the first experiment the inoculation of pure cultures of the two spirochetes, one vibrio (VB5) and one of the presumptive *Bacteroides* strains (B6), gave negative results. Each of the other 7 strains produced a distinct abscess in one or both inoculated animals. Three of these strains (F12, F28 and B13) were reisolated from the exudates, and in the other 4 the inoculated organism could be seen in the exudate microscopically. Transfer of exudate from each of the

TABLE 1.—*Results of first experiment on recombination of the fusospirochetal flora.*

Groups	Cultures inoculated											Responses in guinea pigs
	TM11	TM8	F12	F28	S3	S20	VB4	VB5	B6	B13	L1	
(a) Individual cultures	√											0; 0
		√										0; 0
			√									0; +R
				√								+R; ±
					√							0; +*
						√						0; ±*
							√					±*; ±*
								√				0; 0
									√			0; 0
										√		±* T = ±; +R T = 0
											√	0; ±*
(b) Groups of 4 cultures	√		√		√		√					± (no spirochetes); + (full flora) T = 0
	√		√		√				√			0; ± (full flora) T = 0
	√		√				√		√			0; 0
	√				√		√		√			± (no spirochetes) T = ±; + (full flora)
			√		√		√		√			0; ± T = 0
		√		√		√		√				± (no spirochetes); 0
		√		√		√				√		± (no spirochetes); 0
		√		√				√		√		0; + (full flora) T = 0
				√		√		√		√		0; 0
(c) Groups of 5 cultures	√		√		√		√		√			0; 0
		√		√		√		√		√		0; 0
(d) Groups of 6 cultures	√		√		√		√		√		√	± (no spirochetes) T = 0; ± (no spirochetes); ± (no spirochetes)
		√		√		√		√		√	√	0; 0; 0
(e) 11 cultures	√	√	√	√	√	√	√	√	√	√	√	0; + (no spirochetes); ± (full flora) T = ±; ± (full flora) T = 0

Key to Responses: 0, no abscess formation; ±, small abscess with little exudate; +, moderate abscess with fluid pus; R, culture reisolated from lesion; *, organism seen in exudate but not reisolated; T = exudate transferred to a fresh guinea pig, with the result immediately following. Individual animal responses are separated by semicolons. "No spirochetes" and "full flora" refer to darkfield observations of exudates.

TABLE 2.—*Results of second experiment on recombination of the fusospirochetal flora.*

Groups	Cultures inoculated										Responses in guinea pigs
	TM11	F12	B13	F26	VB2	VB4	VB5	VB6	S20	S17	
(a) Individual cultures	√										0
		√									±; +*
			√								±; +*
				√							0; ±
					√						0; 0
						√					0; ±
							√				0; ±*
								√			±; ±*
									√		0; ±*
(b) Groups of 2 cultures	√	√									± (F); ± (F)
	√				√						0; ± (VB)
	√									√	0; ± (S)
		√			√						± (F); ± (both)
		√								√	± (both); ± (both)
					√					√	0; ± (both)
(c) Groups of 3 cultures	√	√			√						±; ± (no spirochetes)
	√	√								√	± (no spirochetes); ± (no spirochetes)
		√			√					√	+ (all); ± (all)
(d) Groups of 4 cultures	√	√	√		√						± (no spirochetes); ± (no spirochetes)
	√	√	√					√			± (no spirochetes); ± (no spirochetes)
	√	√			√					√	± (no spirochetes); ± (no spirochetes)
	√	√						√		√	± (no spirochetes); ± (no spirochetes)
	√	√			√				√		± (no spirochetes); ± (no spirochetes)
	√	√		√	√						0; ± (no spirochetes)
	√	√				√		√			0; ±
	√	√	√			√				√	± (no spirochetes); ± (no spirochetes)
	√	√				√			√		0; ± (no spirochetes)
	√	√		√		√					0; ±

For key to responses, see table 1. Additional notations: (F), (VB), (S), (both), (all) refer to organisms seen under darkfield in exudates.

animals inoculated with strain B13 failed to demonstrate satisfactory transmissibility of the infection.

Inoculation of combinations of 4 or more strains in the first experiment (table 1) yielded lesions which on the whole were neither larger nor more characteristic of fusospirochetal infection than those obtained with single pure cultures. Whereas typical lesions obtained with fusospirochetal passage exudate regularly attain a diameter of 3 cm or more within the experimental period employed, and often yield 5 ml or more of exudate, none of the lesions in this series exceeded a diameter of 1.5 cm, and none yielded more than 0.5 ml of exudate. These lesions, moreover, showed little evidence of necrosis, and their exudates lacked the foul odor characteristic of fusospirochetal infections. Although in some instances the exudates showed under the darkfield microscope all the morphological types present in the inoculum, these organisms were never found in characteristic abundance. When transferred to fresh guinea pigs these exudates yielded either negative results or lesions that were less marked or less characteristic than that from which the exudate had been drawn.

In the second experiment, summarized in table 2, the results were still less typical of fusospirochetal infection. The most marked responses were again obtained in animals inoculated with strains F12 or B13 alone. Some of the animals inoculated with combinations of 2 or 3 cultures showed all the inoculated forms in the lesion exudates, but in this experiment spirochetes could not be found in any of the exudates.

DISCUSSION

It is apparent from these experiments that something more is required for the experimental reproduction of typical fusospirochetal infection in guinea pigs than the mere recombination of spirochetes, fusiform bacilli, vibrios, streptococci and other bacteria isolated from human gingival and periodontal disease. The absent factors or conditions may have been among the following:

1. One or more essential types of microorganisms may have been lacking. The two strains of spirochetes and the two of *Fusobacterium* seem to have corresponded with those used successfully in earlier studies,[11,12] particularly since the specifications do not seem to have been exacting for either. It may be noted that both strains of *Fusobacterium* manifested a low degree of independent pathogenicity, both having been reisolated from lesions induced with single pure cultures. Among the 4 anaerobic vibrios used, none showed the greening on blood agar described by the previous workers,[12] two of these strains (VB2 and VB5) were completely lacking in independent pathogenicity, and neither of the others yielded lesions comparable in severity with those described by these workers or by Smith.[11] From the data of these two groups of workers the requirement for a streptococcus likewise seems equivocal; and if the reported findings of Kritchewski and Seguin[9] are also accepted, it would seem very doubtful that the experiments here reported failed because none of the 3 streptococci used was appropriate. The further addition of two anaerobic gramnegative bacilli, one of which (strain B13) showed an independent pathogenic effect similar to that of the strains of *Fusobacterium*, and of one strain of *Leptotrichia*, did not appreciably enhance the pathogenicity of these combinations.

2. One or more of the cultures used may have been present in the inoculums in insufficient concentration. While neither Kritchewski and Seguin[9] nor

Smith[11] presented quantitative data on these experiments, the reports of both suggest that the inoculum required for infection is not large. The concentration of cultures used in this work exceeded those used by Proske and Sayers[12] for all the types except spirochetes. The attainment of higher concentrations was prevented in these studies by the presence of agar in the broth cultures, which interfered with effective centrifugation, and by inability to obtain consistent growth of spirochetes in a fluid medium. The latter difficulty is considered more fully in another report.[16] That the present results may have depended on the use of too few spirochetes is suggested (a) by the consistent failure of the spirochete cultures to yield lesions when inoculated individually; (b) by the finding in the first experiment that increasing numbers of types in the inoculum, with correspondingly decreasing concentrations of each type, yielded decreasing responses; and (c) by the consistently poor results obtained in experiment 2, in which the spirochete suspension was light and spirochetes failed to appear in the exudates.

3. Among other possible explanations for these findings are: (a) one or more of the cultures may have undergone attenuation of virulence during the period of cultivation or of preparation of the inoculum; (b) there may have been mutual antagonism among the recombined cultures; or (c) one or more microorganisms in addition to those studied, or another pathogenic form, e.g., a virus, may be required for the production of fusospirochetal infection.

These considerations form the basis of studies which are reported separately.[13,14]

SUMMARY

Attempts were made to determine the pathogenicity of 15 cultures of anaerobic bacteria derived from the human mouth, the cultures having been concentrated and inoculated into guinea pigs individually and in selected combinations of 2, 3, 4, 5, 6 and 11. Included were spirochetes, *Fusobacterium*, vibrios, streptococci, presumptive *Bacteroides* and a *Leptotrichia*, all of which grew only under anaerobic conditions. The cultures of spirochetes could be grown abundantly in an agar medium but only irregularly in an agar-free broth, so that with one exception mass cultures of these organisms were unsuccessful. Guinea pigs inoculated with three individual cultures (two *Fusobacterium* and one presumptive *Bacteroides*) showed small abscesses from which these cultures were reisolated. In certain other instances the full inoculated flora could be identified microscopically in the lesion exudates. The experimental lesions were all smaller than typical fusospirochetal abscesses, showed less abundant exudates lacking the typical fusospirochetal odor, contained lower concentrations of microorganisms, and could not be effectively transmitted to fresh guinea pigs. The findings are discussed in relation to those of previous workers.

STUDIES OF FUSOSPIROCHETAL INFECTION

II. ANALYSIS AND ATTEMPTED QUANTITATIVE RECOMBINATION OF THE FLORA OF FUSOSPIROCHETAL INFECTION AFTER REPEATED GUINEA PIG PASSAGE

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In a previous study[1] it was reported that typical fusospirochetal infection could not be reproduced in guinea pigs with combinations of pure cultures of spirochetes, fusiform bacilli, vibrios, streptococci and other bacteria isolated from gingival or periodontal diseases. The lesions that resulted from inoculation of these cultures separately and in various combinations were small and lacked several of the characteristics of the infection, as it is regularly elicited with unpurified guinea pig passage exudates. These findings were thought to be due to one or more of the following conditions: (a) essential members of the fusospirochetal flora may have been lacking (b) the preparations used may have been insufficiently concentrated or (c) the cultures may have been insufficiently virulent or mutually antagonistic. The present report deals with experiments designed to supply these missing conditions. It is based on studies with a single guinea pig passage "strain" of fusospirochetal infection. The infectivity of this strain was determined by titration of exudate in guinea pigs, and the numbers of each contained morphological type of microorganism in the infective dose determined by microscopic count. The exudate was then analyzed by cultural means in an effort to isolate all its component microorganisms. Mass pure cultures were then prepared, recombined as nearly as possible in the estimated concentration and inoculated into guinea pigs.

METHODS

The source material for these studies was a suspension of gingival scrapings from a case of Vincent's gingivitis (M.P.). The suspension, in extract broth, was inoculated subcutaneously into guinea pigs,[2,3] and exudate from the resulting lesions passed serially through fresh guinea pigs. Exudate was aspirated only from nondraining localized lesions on the 5th to the 9th day after inoculation, and either passed directly to fresh animals or preserved by freezing in solid carbon dioxide.[4] Strict aseptic technique was adhered to in order to avoid the possible introduction of extraneous microorganisms into the exudates. Routine aerobic cultures of passage exudates on sheep blood agar-streaked plates showed the presence of a greening streptococcus as the only aerobic microorganism present.

For titration of infectivity only, a second passage strain of fusospirochetal infection was used in addition to the M.P. strain. The second strain (C.S.), derived from a case of marginal gingivitis, was handled by the same procedure.

Exudate aspirated from 3 guinea pigs of the 8th passage was pooled for titration of the M.P. strain, while a pool of 2 guinea pig exudates of the 10th passage was used for the C.S. strain. Groups of 4 guinea pigs, 250 to 350 g in weight, were inoculated subcutaneously in the left groin with, respectively, 0.8, 0.4, 0.2 and 1 ml of undiluted exudate, and 0.1 ml of the following dilutions in extract broth: 1:2, 1:4, 1:8, 1:16, 1:32,

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1. Rosebury, T., Clark, A. R., Engel, S. G. and Tergis, F. 1950, J. Infect. Dis. **87**: 217–225.
2. Rosebury, T. and Foley, G. 1939, J. Am. Dent. A. **26**: 1798.
3. Rosebury, T., Macdonald, J. B., Clark, A. R. and Frances, S. Dent. Research. In press.
4. Rosebury, T. and Frances, S. Oral Surg., Oral Med. & Oral Path. In press.

and 1:64. With the C.S. strain an additional group of guinea pigs received 0.1 ml of a 1:128 dilution. The animals were examined daily and dead animals autopsied. Surviving animals were sacrificed and autopsied on the 11th day after inoculation. Infected animals all showed typical fusospirochetal lesions with three exceptions in which intercurrent infections occurred; these animals have been excluded from the tabulations. Animals were recorded as infected or dead, and LD_{50} and ID_{50} values were estimated from the data by the method of Reed and Muench.[5]

The concentration of microorganisms was estimated, both in the pooled exudate used for the guinea pig titration and in the culture suspensions used for the recombination experiments, as follows. Two or more dilutions of each sample were used for each determination. Aliquots of each dilution were mixed with an equal volume of finger blood in a white-cell pipette and blood films prepared. Blood from the same subject, with a count of 4.5×10^9 erythrocytes per ml, was used throughout. For spirochetes the slides were fixed by immersion in 10% formalin for 1 minute, dried in air and stained by the Victoria blue method of Goldsworthy and Ward.[6] For all other organisms separate slides were fixed by flooding them with methyl alcohol, flaming and allowing the alcohol to burn off, and stained by the Kopeloff and Beerman[7] modification of Gram's method. For the exudate samples differential counts were made of the following morphological categories, corresponding approximately to the cultures isolated from the M.P. strain as described below: spirochetes (undifferentiated); small gram-negative fusiform rods; curved gram-negative rods ("vibrios"); other gram-negative rods; small gram-positive rods; gram-positive bent rods; and streptococci. Counts were estimated as averages for the slides of each sample of organisms per 1000 red cells $\times$ dilution $\times 4.5 \times 10^6$ = organisms per ml.

For isolation of the microorganisms in M.P. exudates the mediums and methods in a previous report[1] were used. All cultures were derived from anaerobic plates seeded with exudates of 8th, 9th or 10th guinea pig passages. The earliest available pure subculture (or, with spirochetes, the earliest bacteria-free subculture)[8] of each was stored in solid carbon dioxide.[4] The following 15 cultures were selected for the recombination experiments out of 24, which included replicate cultures or closely related forms.*

Spirochetes.—TM13. From a demarked haze[8] in a 6-day AFK plate; originally tightly wound but very variable in subcultures, with many intermediate forms.

TM13b. From a haze slightly less clearly demarked than the preceding, in a 5-day AFK plate. Tightly wound, variable.

TM13d. From an intermediate haze in a 5-day SUK plate; intermediate, variable.

Fusobacterium.—F39a. From a smooth colony with internal markings, on a 3-day gentian violet agar plate. Small granular fusiform rods, gram-negative, nonmotile. No growth on aerobic blood agar. Acid in dextrose, no acid in sucrose or mannite. Produced indol, ammonia, urease, and small amounts of hydrogen sulfide; gas formed in thioglycollate-1% agar shake cultures; nitrate not reduced.

Vibrios.—VB7a. Separated from spirochetes, probably incompletely, after 4 passages in AFK tube cultures, by growth in thioglycollate broth with 10% ascitic fluid, and in enriched veal infusion broth.[1] No growth on the surface of solid mediums aerobically or anaerobically; light growth in the fluid mediums mentioned. Curved rods, 0.4 to 0.6×5 to 7 μ, gram-negative, active tumbling motility. Very pleomorphic in thioglycollate mediums. Acid in dextrose and sucrose; no acid in mannite. No growth in other test mediums.

VB8. From a smooth, low-raised translucent colony, less than 1 mm in diameter, on a 4-day blood agar plate. Small gram-negative rods, straight or slightly curved, some with pointed ends, motile. No growth aerobically on blood agar. Growth in thioglycollate broth and in enriched veal infusion broth. Acid in dextrose and sucrose, slight acid in mannite. No growth in other test mediums.

VB9. From a 5-day culture on blood agar containing 50 units of bacitracin; probably identical with VB8.

B22. From a 1.5 mm smooth convex colony with opaque center on a 7-day blood agar plate. Short, slender straight gram-negative rods and coccoidal forms, some in short chains, sluggishly motile. Moderate growth in thioglycollate broth and in enriched veal infusion broth. No growth

5. Reed, L. J. and Muench, H. 1938, Am. J. Hyg. **27**: 493.
6. Goldsworthy, N. E. and Ward, H. K. 1942, J. Path. & Bact. **54**: 382.
7. Kopeloff, N. and Beerman, P. 1922, J. Infect. Dis. **31**: 480.
8. Rosebury, T., Macdonald, J. B., Ellison, S. A. and Engel, S. G. Oral Surg., Oral Med. & Oral Path. In press.

* For further biochemical data on these strains see the next paper in this series.

aerobically on blood agar. Acid in dextrose; slight acid in sucrose and mannite. Produced urease and ammonia. No growth in other test mediums.

Nonmotile gram-negative anaerobic rods.—F41. From a 1 mm smooth translucent granular colony on a 3-day blood agar plate. Gram-negative rods, filaments and swollen (funduliform) elements, nonmotile. No growth aerobically on blood agar. Poor growth in thioglycollate broth and in enriched veal infusion broth. Dextrose, sucrose and mannite not fermented; no indol, ammonia or urease produced.

B23. From a 1 mm rough, convex colony on a 7-day blood agar plate. Small gram-negative rods, some straight, some curved, some ovoid; nonmotile. No growth aerobically on blood agar. Moderate growth in thioglycollate broth and in enriched veal infusion broth. Acid in dextrose, sucrose and mannite. Produced ammonia and urease. No growth in other test mediums.

Nonmotile gram-positive anaerobic rods.—R28. From a 0.5 mm, high, convex, yellow, smooth, opaque colony on a 3-day blood agar plate. Slender gram-positive rods, nonmotile, characteristically bent but unbranched; many V- and Y-groups. No growth aerobically on blood agar. Heavy growth in thioglycollate broth and in enriched veal infusion broth. Acid in dextrose, sucrose and mannite; gas in thioglycollate agar shake cultures; no indol, hydrogen sulfide, ammonia or urease produced; nitrate not reduced.

R29. From a 0.5 mm, low, convex, smooth, graying translucent colony on a 3-day blood agar plate. Small, straight, uniform, gram-positive rods, some in chains; nonmotile. No growth aerobically on blood agar. Moderate growth in thioglycollate broth and in enriched veal infusion broth. Acid in dextrose, sucrose and mannite. Produced ammonia and urease, no indol, no hydrogen sulfide.

Streptococci.—S23. From a 1 mm, round, low convex, translucent granular colony on a 3-day (anaerobic) blood agar plate. Gram-positive diplococci and streptococci, nonmotile. Growth aerobically on blood agar, with greening. Heavy growth in thioglycollate and other broth mediums. Acid in dextrose, sucrose and mannite. Produced ammonia; no indol, hydrogen sulfide or urease; nitrate not reduced.

S26. From a 0.5 mm, grayish, smooth, translucent colony on a 3-day blood plate. Large gram-positive diplococci and streptococci, nonmotile. No growth aerobically on blood agar. Moderate growth in thioglycollate broth and in enriched veal infusion broth. Acid in dextrose, sucrose and mannite. No indol, hydrogen sulfide, ammonia or urease produced; nitrate not reduced.

S27. From a minute (less than 0.5 mm), smooth, transparent colony on a 3-day blood plate. Small gram-positive diplococci and streptococci, nonmotile. No growth aerobically on blood agar. Light growth in thioglycollate broth and in enriched veal infusion broth. No acid in dextrose, sucrose or mannite. Produced hydrogen sulfide, ammonia and urease, no indol; nitrate not reduced.

Procedure for recombination experiments.—During the course of these experiments the studies on spirochetes reported elsewhere[8] were in progress, and it was decided on the basis of current findings to prepare the spirochete cultures by growth in 125 ml flasks of fluid Proske-Sayers AFK broth ("FAFK") and alternately in the same medium containing 0.1% agar. Duplicate flasks of each medium were used for each strain. Freshly thawed samples of each spirochete culture were inoculated first into tubes of the 0.1% agar medium and incubated 7 days at 37 C. Selected cultures of each strain showing abundant pure growth of spirochetes by darkfield examination were transferred once more through 7-day cultures in tubes of the same medium and then seeded from the heaviest culture of each strain into the flasks, which were incubated in anaerobic jars for 7 days at 37 C. Parallel tube cultures in the FAFK medium showed distinctly less marked growth than those in the 0.1% agar medium and were not used. The FAFK flask cultures, however, all showed abundant growth (100 to 200 spirochetes per oil-immersion field in the whole broth) and were selected for concentration.

Preliminary experiments with the other cultures led to choice of the following mediums and growth schedules. Fluid mediums were prepared for mass cultures in 125 ml flasks as in previous experiments.[1] In addition, sheep blood agar was prepared in 4 oz. screw-cap bottles, incubated for sterility at 37 C aerobically for 24 hours, then in anaerobic jars for 48 hours before inoculation. The 3 streptococci, and strains F39a and R28 were grown in duplicate for 3 days in enriched veal infusion broth and in thioglycollate broth containing 10% of ascitic fluid. Strain VB7a was grown for 3 days in the former medium only. Strains B23, F41 and R29 were grown for 5 days in the same two mediums, and strains VB8, VB9 and B22 were grown for 5 days in enriched veal infusion broth and as surface cultures on blood agar. All cultures were grown first in tubes of the respective mediums as noted above, seeded from freshly thawed samples and incubated for the indicated 3- or 5-day periods. The most favorable seed cultures were then inoculated into flasks of broth, or streaked heavily on blood agar in bottles, or both. At each transfer of all

cultures, streaked blood agar plates were incubated aerobically as checks on purity, in addition to routine Gram stains and darkfield preparations. All cultures, including spirochetes, were scheduled in such a way that the final mass cultures were ready for harvesting on the same day.

The mass cultures showing most abundant growth and therefore selected for recombination were: in thioglycollate-ascitic fluid broth, strains S23, S26, S27, F39a, R28, R29 and B23; in enriched veal infusion broth, strains F41 and VB7a; and on blood agar, strains of VB8, VB9 and B22. The contents of each flask, including spirochete cultures, were transferred to two 100 ml centrifuge tubes, and the growth from each bottle washed off with 5 ml of the supernatant from centrifuged sterile thioglycollate broth. After centrifugation at approximately 1800 r.p.m. for 1 hour, the sediments were resuspended in minimal volumes of broth, using centrifuged sterile thioglycollate broth for cultures grown in that medium or on blood agar, and the supernatant from the individual cultures for the others. Pooled suspensions for each strain were then counted by the method previously noted, using dilutions in 0.85% sodium chloride containing 1% dextrose. The counts ranged from 2.3 to 28.4×10^9 organisms per ml, except for strain F41, which grew poorly and yielded only 0.2×10^9 organisms per ml. The lower counts were associated either with poor growth or with the presence of agar in the medium, which prevented effective centrifugation. The 3 strains of spirochetes, which grew well and were centrifuged from agar-free FAFK broth, gave yields per ml ($\times 10^9$) of 11.7 (TM13), 28.4 (TM13b) and 22.0 (TM13d). From the counts the volumes were calculated for each suspension to make up suitable amounts of mixtures such that an animal inoculum of 1 ml or less would contain approximately the required number of each type. As is shown in table 3, an adequate approximation of the concentration estimated as required for infection was obtained with all cultures except the streptococci. Suspensions of these organisms were light in proportion to the high concentrations required. Hence, to avoid excessive dilution of the recombination mixtures, approximately half the number estimated as required in the LD_{50} was employed. Twice the number of strain R29 estimated as required was included in the first 3 experimental mixtures by error.

Since the operations of harvesting, concentrating and counting the cultures consumed two days, the suspensions, after being prepared, were stored in a refrigerator at 4 C for approximately 40 hours before being recombined, the animal inoculations being made on the second day after harvesting.

Groups of 6 guinea pigs were inoculated with each of 7 preparations, including 2 intended as controls. The first preparation comprised all 15 strains suspended in extract broth; the inoculum was 0.85 ml. The second mixture was the same as the first except that, after having been prepared, it was recentrifuged at 1800 r.p.m. for 1 hour and the sediment resuspended in an equivalent volume of a sterile Seitz filtrate of pooled guinea pig fusospirochetal exudate; the inoculum was 0.85 ml. The next three groups received extract broth suspensions corresponding with the first preparation except that the following strains were omitted: in group 3, S23 (the aerobic streptococcus), inoculum 1 ml; in group 4, B23, F41, R28 and R29, inoculum 0.6 ml; and in group 5, all 5 cultures omitted in the 2 previous groups, inoculum 0.8 ml. Group 6 was inoculated with 0.1 ml of freshly thawed exudate (M.P., 9th passage). This inoculum, as indicated below, was estimated as roughly equivalent in kinds and concentrations of microorganisms to the recombined suspension used in groups 1 and 2. Group 7 received 1 ml of a mixture of sediments prepared by centrifuging sterile thioglycollate and enriched veal infusion broths and combining them in proportions equivalent to those of Group 1.

Guinea pigs weighing from 300 to 350 g were inoculated subcutaneously in the left groin, using added traumatization with the injection needle for all animals except the exudate controls, group 6. The animals were examined daily and, in the experimental groups, beginning with the 5th day after inoculation, all lesions with evidence of fluctuation were aspirated, the exudate examined by darkfield illumination and, where the results warranted, passed to a fresh animal. Transfers were not undertaken for group 6, in which typical fusospirochetal responses appeared, nor in group 7, in which no aspirable lesions resulted.

RESULTS

The results of the two guinea pig titrations, with exudates of the M.P. and C.S. strains, respectively, are given in tables 1 and 2. These experiments represented attempts at greater precision than was achieved in an earlier study.[9] As would be expected, however, with inoculums of low infectivity, the variability

9. Shpuntoff, H. and Rosebury, T. 1949, J. Dent. Research, **28**: 7.

of the findings is still high—indeed too high for adequate statistical treatment. There is nevertheless a clear indication in both tables of decreasing infectivity and lethality with increasing dilution; and the close agreement of estimated 50% lethal and infective doses for the two strains suggests their rough reliability. In both series it may be noted that infectivity seemed to fall off sharply between the 1:16 and the 1:32 dilutions. The single infection that occurred in each series at a higher dilution (1:64) was a very small but nevertheless typical fusospirochetal abscess. Death, on the other hand, which in this instance is thought to result from overwhelming infection, was much more scattered in its occurrence and may be assumed to have reflected accidents of distribution of the inoculum or of immediate host response as factors of greater weight than dosage in the tested range. These results seemed acceptable for the purposes of this study, and the rounded value, 0.1 ml of undiluted exudate, was taken as the LD_{50}. The attempt was therefore made to match this inoculum as closely as possible with recombined cultures. The fact that 1/25 of the indicated inoculum could still be expected to yield typical fusospirochetal infection in 50% of inoculated animals seemed to provide a satisfactory margin of safety.

TABLE 1.—*Titration of fusospirochetal exudate in guinea pigs, series M.P.*

Volume inoculated	Dilution	Responses of guinea pigs				
		Negative	Localized abscess	Extension	Infected	Dead
					Total	Total
ml						
0.8	0	0	0	4	4/4	4/4
0.4	0	0	2	2	4/4	1/4
0.2	0	0	2	1	3/3	0/3
0.1	0	0	2	2	4/4	2/4
0.1	1:2	0	0	3	3/3	2/3
0.1	1:4	0	2	2	4/4	1/4
0.1	1:8	0	3	1	4/4	1/4
0.1	1:16	0	4	0	4/4	0/4
0.1	1:32	4	0	0	0/4	0/4
0.1	1:64	3	1	0	1/4	0/4

8th passage exudate pooled from 3 animals, diluted in extract broth. Inoculations subcutaneous.
LD_{50} = (approximately) 0.1 ml of 1:1.2 dilution.
ID_{50} = (approximately) 0.1 ml of 1:25 dilution.

TABLE 2.—*Titration of fusospirochetal exudate in guinea pigs, series C.S.*

Volume inoculated	Dilution	Responses of guinea pigs				
		Negative	Localized abscess	Extension	Infected	Dead
					Total	Total
ml						
0.8	0	0	0	4	4/4	3/4
0.4	0	0	0	4	4/4	4/4
0.2	0	0	3	1	4/4	1/4
0.1	0	0	1	3	4/4	3/4
0.1	1:2	0	2	2	4/4	2/4
0.1	1:4	0	3	1	4/4	1/4
0.1	1:8	0	2	1	3/3	1/3
0.1	1:16	0	4	0	4/4	0/4
0.1	1:32	4	0	0	0/4	0/4
0.1	1:64	3	1	0	1/4	0/4
0.1	1:128	4	0	0	0/4	0/4

10th passage exudate pooled from 2 animals, diluted in extract broth. Inoculations subcutaneous.
LD_{50} = (approximately) 0.1 ml of 1:1.3 dilution.
ID_{50} = (approximately) 0.1 ml of 1:25 dilution.

The values given in the first column of table 3 represent corrected averages based on counts of the exudate used for the M.P. titration. Two values have been corrected mutually, those for "vibrios" and for "gram-negative rods." Curved gram-negative rods identified as vibrios in the stained slides were estimated as 80×10^6 (per 0.1 ml), while other gram-negative rods were estimated as 440×10^6. To allow for the fact that the "vibrio" group of cultures included straight as well as curved rods, one quarter of the second value was added arbitrarily to the first, giving the values in the table. It seems worthy of note that the total LD_{50} of fusospirochetal exudate for the guinea pig as estimated is approximately 8×10^9 organisms.

The remaining columns of table 3 present the estimated numbers of each culture suspension in the recombined mixtures as they were inoculated into guinea pigs. The volume inoculated into each animal was held at or below 1 ml, while the variations from group to group depended on the use of volumes of each suspension that were readily measurable with pipettes.

The results in the inoculated animals

TABLE 3.—*Estimated numbers of organisms in the LD_{50} of M.P. exudate and in the recombined culture mixtures as used for animal inoculation.*

		LD_{50} of exudate	Culture mixtures for experiments			
			1–2	3	4	5
	Volume, ml	0.1	0.85	1.0	0.6	0.8
	Strain		*numbers* $\times 10^8$			
Spirochetes	TM13		490	480	480	500
	TM13b		510	500	500	520
	TM13d		520	520	510	540
		1700	1520	1500	1490	1560
Fusiform bacilli	F39a	230	230	220	220	230
Vibrios	VB7a		30	30	30	30
	VB8		40	40	40	40
	VB9		40	40	40	40
	B22		50	50	50	50
		190	160	160	160	160
Streptococci	S23		750		730	
	S26		730	1100	710	1100
	S27		690	1050	680	1100
		5400	2170	2150	2120	2200
Gram-negative rods	B23		320	320		
	F41		10	10		
		330	330	330		
Gram-positive straight rods	R29	130	260	260		
Gram-positive bent rods	R28	110	100	100		

are summarized in table 4. Four animals in group 1, and one animal in each of groups 2, 3 and 5, developed a localized abscess not greater than 1.5 cm in diameter at its peak, and containing exudate in which all the inoculated morphological types, including motile spirochetes, could be demonstrated by darkfield examination. These lesions either drained spontaneously or were aspirated on the 5th to the 8th day after inoculation. None showed the necrosis or the foul odor typical of fusospirochetal infection. When transferred, the second animal showed a less marked reaction than the first. Spirochetes were not found in the exudates of any of the passage animals.

While differences in animal response among the 5 experimental groups were small and may not be significant, the most marked response was observed in group 1, in which the inoculated mixture contained all 15 cultures suspended in

TABLE 4.—*Results of guinea pigs inoculated with recombined mixtures of cultures derived from the M.P. exudate strain of fusospirochetal infection.*

Experimental groups						
1	2	3	4	5	6	7
15 cultures in broth	15 cultures in exudate-filtrate	14 cultures in broth	11 cultures in broth	10 cultures in broth	Exudate controls	Culture sediment controls
+, T = ±	+, T = ±	+, T = ±	±, T = 0	+, T = 0	++++d	±0
+, T = ±	±, T = ±	±, T = 0	±, T = 0	±	++++d	±0
+	±	±, T = 0	±	±	++++d	±0
+	±	±	±	±	++L	0
±, T = ±	±	±	±0	±	++L	0
±	±0	±	±0	±0	++L	0

Key: 0, no response; ±0, minimal response, few or no organisms found (no spirochetes); ±, moderate response, organisms numerous, no spirochetes; +, moderate response with full flora including spirochetes; ++L, typical localized fusospirochetal infection; ++++d, typical fatal spreading fusospirochetal infection; T = ±, or T = 0, transferred to a fresh guinea pig with the indicated response.

broth. Substitution, in group 2, of fusospirochetal exudate filtrate as suspending medium in place of broth did not enhance this response. Elimination from the mixture of the aerobic streptococcus (group 3) of the gram-positive and gram-negative nonmotile bacilli other than *Fusobacterium* (group 4) or of both aerobic streptococci and these bacilli (group 5) gave responses of slightly diminished intensity.

The effects of inoculation of M.P. exudate (group 6), incorporating an infective dose estimated to be equivalent in kind and in concentration to that of groups 1 and 2, were unmistakeably more marked than those observed in any of the experimental groups. Three of these animals died on the 3rd, 5th and 7th day, respectively, after inoculation, all with the typical picture of a foul, necrotizing infection spreading widely through the subcutaneous tissues. The other three developed localized abscesses from 2.5 to 4 cm in diameter, which on aspiration yielded typical abundant fusospirochetal exudates.

The sediment controls (group 7) showed slight swellings at the point of inoculation, persisting for a week as small indurated areas in three instances, but without abscess formation.

DISCUSSION

Analysis of a typical fusospirochetal infection after passage through guinea pigs, both quantitatively in terms of infectivity and qualitatively in terms of the kinds of microorganisms suspended in it, is not known to have been reported previously. Since the 15 cultures obtained from passage exudates, when recombined in concentrations that seemed well within the limits of the infective dose, failed to elicit responses such as were obtained with exudate itself, it is necessary to examine the data for possible reasons underlying this result.

Unlike the cultures used in the previous study,[1] which were derived from several sources and may have represented an unsuitable or incompatible mixture, those used in the present study were all obtained from one strain of infective exudate. Mutual incompatibility among the cultures would thus seem unlikely. While the 15 cultures seemed to embrace all the morphological varieties of microorganisms observed in exudates by darkfield examination or in stained smears, the possibility is not excluded that one or more forms essenential for infectivity was not obtained in the cultures. If an infective factor present in whole exudate was missing from the culture mixtures, however, it is evident that sterile filtrate of exudate did not supply it, as is indicated by the results with group 2 (table 4). Findings in further studies with additional cultivable forms from the M.P. exudate strain, and with sterile exudate filtrate and the supernatant fluid from centrifuged exudate, are given in the next report in this series.[10]

The data on infectivity of exudate in this report strengthen the view suggested previously[1] that the earlier failure may have been due to insufficient spirochetes or other organisms in the culture mixtures. It seems unlikely, however, that a similar explanation can apply to the present experiments, in which the culture concentrations were well within the 25-fold range of permissible variation indicated by the difference between exudate LD_{50} and ID_{50} values. It is, of course, true that these estimated values were based on total counts, which failed to distinguish between viable and nonviable organisms, and that the proportion of nonviable forms may have been significantly higher in the cultures, in view of

10. Rosebury, T., Clark, A. R., Macdonald, J. B. and O'Connell, D. C. 1950, J. Infect. Dis. **87**: 234–248.

the waiting period necessitated by the work of harvesting and counting the cultures. Data bearing on this question are also given in the next report.[10]

The related possibility of impaired virulence of the cultures incident to their manipulation can hardly be tested in the absence of means for demonstrating the pathogenicity of individual or combined cultures. The manipulation to which the cultures used in the present study were subjected, and the time required for it, were held to a minimum consistent with purity of the cultures and the need to obtain them in adequate concentration and amount. The effects of storage in solid carbon dioxide on the virulence of these organisms is not known, but it seems unlikely that such storage could have been more detrimental than any other means of holding cultures during the unavoidable waiting intervals between isolation and their inoculation into the recombination mediums. It may be noted that whole exudates withstand storage in dry ice well.[4] Confirmation of this finding is indicated by comparison of the results obtained in this study with freshly thawed exudate (group 6, table 4) and with fresh exudate of the same strain (table 1).

Another condition applied in this study which may have influenced the results was the larger volume used with the culture mixtures (0.6 to 1 ml) as animal inoculum compared with inoculum of exudate (0.1 ml). Tests of this possibility, and other studies with M.P. exudates and with cultures derived from them, are given in the subsequent report.[10]

SUMMARY

Subcutaneous inoculation into guinea pigs of graded amounts of each of two "strains" of fresh fusospirochetal exudate after repeated guinea pig passage, using twofold dilution intervals, indicated that the LD_{50} is slightly less than 0.1 ml of undiluted exudate, while the ID_{50} is approximately 0.1 ml of a 1:25 dilution. The two strains showed good agreement in these values. Microscopic counts of one of these exudates (strain M.P.) indicated that the LD_{50} for the guinea pig contained approximately 8×10^9 microorganisms, made up as follows (all $\times 10^9$): spirochetes, 1.7; fusiform bacilli, 0.23; vibrios, 0.08 to 0.19; streptococci, 5.4; gram-negative bacilli, 0.44 to 0.33; and gram-positive bacilli of at least two varieties, 0.24.

Aerobic blood agar cultures of M.P. exudate revealed only a greening streptococcus. This organism and 14 anaerobic forms apparently embracing the morphological varieties seen microscopically were isolated from M.P. exudates and preserved by storage in solid carbon dioxide. Mass cultures of the 15 strains, prepared after minimal subculturing, were recombined and inoculated subcutaneously into guinea pigs, so that each animal received in a volume of 0.6 to 1 ml the numbers of each organism corresponding roughly to the LD_{50} as estimated for exudate. Five groups of guinea pigs received mixtures containing from 10 to all 15 of the cultures. These mixtures yielded small, nontransmissible abscesses, some of which seemed to contain the full flora, but none was a typical fusospirochetal response. The use of sterile filtrate of fusospirochetal exudate as suspending medium for one of the mixtures of 15 cultures did not enhance its infectivity. A control group of guinea pigs inoculated with 0.1 ml of M.P. exudate thawed after solid carbon dioxide storage yielded 3 specific deaths and 6 specific infections out of 6 animals.

STUDIES OF FUSOSPIROCHETAL INFECTION

III. FURTHER STUDIES OF A GUINEA PIG PASSAGE STRAIN OF FUSOSPIROCHETAL INFECTION, INCLUDING THE INFECTIVITY OF STERILE EXUDATE FILTRATES, OF MIXED CULTURES THROUGH TEN TRANSFERS, AND OF RECOMBINED PURE CULTURES

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In two previous reports[1,2] it was noted that fusospirochetal infection, although regularly transmissible by passage of exudate through guinea pigs, could not be reproduced with cultures derived either from such exudates or from gingival and periodontal lesions in man that serve as sources of the guinea pig disease. In the second of these papers[2] the infectivity of two guinea pig "strains" of this passage infection was determined by animal titration, and the numbers of microorganisms in the LD_{50} of one of them estimated by differential microscopic counts. Cultures made from this strain yielded 15 apparently distinct microorganisms, including spirochetes, fusiform bacilli, vibrios, streptococci, and both gram-positive and gram-negative nonmotile bacilli, all growing only under anaerobic conditions except for one strain, a greening streptococcus. Recombination of these cultures in numbers roughly equivalent to those estimated in the LD_{50} of exudate elicited in guinea pigs nontransmissible lesions which fell short of reproducing the typical fusospirochetal pattern. Among the possible reasons for this finding were loss of virulence or viability, or both, during the period of manipulation of the cultures, the greater volume of the recombined culture inoculum for guinea pigs (0.6 to 1 ml) as compared with that of exudate (0.1 ml), and the possibility that one or more essential infective factors were missing from the recombined mixtures.

In the present study attempts have been made to throw further light on these questions. The stability of whole exudate on standing has been examined, as has the effect on infectivity of increasing volumes of animal inoculum; and experiments are presented on the infectivity of sterile filtrates of exudate and of the supernatant fluid from centrifuged exudate. In addition, this report presents a study of the infectivity for guinea pigs of mixed cultures prepared by inoculating whole filtrate into spirochete plates[3] or as spot cultures on anaerobic blood agar plates. Similar experiments, apparently involving only one culture generation, have been reported by Hemmens and Harrison.[4]

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1. Rosebury, T., Clark, A. R., Engel, S. G. and Tergis, F. 1950, J. Infect. Dis. **87**: 217–225.
2. Rosebury, T., Clark, A. R., Tergis, F. and Engel, S. G. 1950, J. Infect. Dis. **87**: 226–233.
3. Rosebury, T., Macdonald, J. B., Ellison, S. A. and Engel, S. G. Oral Surg., Oral Med. & Oral Path. In press.
4. Hemmens, E. S. and Harrison, R. W. 1942, J. Infect. Dis. **70**: 131.

Since such cultures were found to be infective, they were transferred serially, with guinea pig inoculation at each step. Additional individual cultures were derived from these mixtures, combined with the previous group of strains,[2] screened to eliminate replicates, and recombined in a further effort to reconstitute infective mixed cultures.

These experiments were all conducted with the single exudate strain M.P., derived originally as gingival scrapings from a case of acute Vincent's gingivitis and maintained by subcutaneous passage through guinea pigs and by storage of exudates in solid carbon dioxide.[5]

EXPERIMENTAL

1. Effects of storage of exudate and of volume of inoculum

Procedure.—Exudate from 3 guinea pig of the 11th passage was thawed after removal from dry ice, pooled, and 0.1 ml inoculated subcutaneously into each of 4 guinea pigs. The pooled exudate was then divided into 4 portions, 3 of which were placed in cotton-stoppered Wassermann tubes in the dark, in the refrigerator (4 C), at room temperature (18 to 24 C) and in the incubator (37 C), respectively. After 1 hour, 6 hours and 24 hours, each of the 3 samples of exudate was again inoculated into 4 guinea pigs, using 0.1 ml by the subcutaneous route.

The 4th portion of the same exudate pool was used for preparation of 1:2, 1:4, 1:8 and 1:16 dilutions in extract broth. Along with 4 additional control guinea pigs, each of which received 0.1 ml of undiluted exudate subcutaneously, these dilutions were inoculated into 4 guinea pigs for each, using volumes, corresponding to the dilutions as given, of 0.2, 0.4, 0.8 and 1.6 ml, so that all 20 guinea pigs received the same amount of exudate.

The guinea pigs in both series were examined over a period of 7 days. On the 4th or 5th day after inoculation one guinea pig of each group in both series, selected as having a suitable lesion, was used for aspiration and passage to 2 fresh guinea pigs (in one instance 4 passage animals were used). The passage animals were again examined over a 7-day period.

5. Rosebury, T. and Frances, S. Oral Surg., Oral Med. & Oral Path. In press.

Results.—Darkfield examination of the stored exudates showed some depression of spirochetal motility after 6 hours at both 4 and 37 C, but not at room temperature. After 24 hours, the great majority of spirochetes in all 3 specimens had become inactive. A few were sluggishly motile in the room temperature specimen, while in the other two tubes the few motile forms present were still vigorously active. Motility of vibrios was not noticeably impaired in any of the samples except the 24-hour sample at 4 C, in which, however, a few of these forms were still active.

Results obtained in inoculated guinea pigs are summarized in tables 1 and 2. In these and subsequent experiments presented in this report, a uniform scheme for grading fusospirochetal lesions has been used, as indicated in the key under table 1. This may be amplified as follows: the ± lesion was an abscess less than 1 cm in diameter, or a nonfluctuating swelling or induration. Fluid was palpable in all the larger lesions: +, a localized abscess 1 to 1.5 cm in diameter; ++ , a larger similar lesion, still well localized; +++, a lesion with fluctuating extension palpable for at least 3 cm, tending to progress slowly or remain stationary, the animal surviving the experimental period; and ++++, a lesion with more marked and progressive extension, fatal within the experimental period.

It will be seen (table 1) that storage of exudate for 6 hours at the 3 temperatures did not seem to impair its infectivity, since both original and passage animals compared favorably with the controls. The same appears to be true of the samples held for 24 hours in the refrigerator and at room temperature. In the 24-hour, 37 C sample a slight decrease of doubtful significance is indicated by the findings.

It is apparent from the data in table 2

TABLE 1.—*Infectivity of fusospirochetal exudate for guinea pigs after standing for different intervals at different temperatures.*

Stored		Responses of guinea pigs					
Temperature	Hours	Original animals				Passage animals (from*)	
4 C	1	++,	++,	++,	++++*	++,	++++
	6	++,	++,	++,	++++*	+++++,	++++
	24	+,*	++,	+++++,	++++	++,	+++
18–24 C	1	+,	++,*	+++,	++++	++,	++
	6	++,	+++,*	+++++,	++++	++,	+++++
	24	+,	++,	++,	++++*	++,	++
37 C	1	++,*	++,	++,	+++	+++,	+++
	6	++,*	++,	++,	++++	+++++,	++++
	24	±,‡	±,	+,	++†	++,	++
Fresh exudate		++,	++,	++,	+++	++,	++

Exudate series M.P.; inoculum 0.1 ml, subcutaneous.
Notations for individual animals are separated by commas.
±, very small local lesion.
+, moderate local lesion.
++, large local lesion.
+++, spreading cellulitis, not fatal in 7 days.
++++, spreading cellulitis, fatal in 7 days or less.
* Exudate transferred on the 4th day after inoculation.
† Exudate transferred on the 5th day after inoculation.
‡ This animal died of intercurrent infection on the 2nd day after inoculation, showing little fusospirochetal response

TABLE 2.—*Effect of volume of inoculum on infectivity of fusospirochetal exudate for guinea pigs.*

Volume of inoculum	Responses of guinea pigs						
	Original animals				Passage animals (from*)		
ml							
0.1	++,	++++,*	++++,	++++	++,	++,	++, +++
0.2	++,	++++,*	++++,	++++	++,	++++	
0.4	+,	++++,*	++++,	++++	++++,	++++	
0.8	++,*	+++,	+++,	+++	++++,	++++	
1.6	++,	++,	++++,*	++++	++,	++	

Exudate series M.P. All inoculums contained 0.1 ml of whole exudate diluted with extract broth to the indicated volumes. Injections subcutaneous.
For key, see table 1.

that volume of inoculum within the limits tested did not alter the response in inoculated guinea pigs.

2. Infectivity of centrifuged and filtered exudate

Procedure.—Exudate from 3 guinea pigs of the 12th passage was thawed after removal from dry ice, pooled, and 1 ml of the pool removed. The remainder (11 ml) was centrifuged under refrigeration at 1800 r.p.m. and the sediment discarded. Two ml of the clear supernatant fluid was removed and the remainder (4 ml) was passed under negative pressure through a Berkefeld N filter into a container surrounded by cracked ice. Animal inoculations were made immediately. Of the aliquot of untreated exudate, 0.1 ml was inoculated subcutaneously into each of 4 guinea pigs. Both filtered and unfiltered supernatant fluids were treated as follows: 0.2 ml was injected subcutaneously into each of 4 guinea pigs; 2 rabbits were inoculated on the scarified cornea with approximately 0.05 ml and 0.1 ml, respectively; and 7 mice were inoculated intracerebrally with 0.03 ml.

Results.—Darkfield examination of the whole exudate showed it to be typical. The unfiltered supernatant showed widely scattered fusiform and other bacilli and cocci. A sheep blood agar plate streaked with filtered supernatant was sterile after 5 days anaerobically at 37 C.

The 4 control guinea pigs inoculated with whole exudate showed typical responses, one +++ and 3 ++++. The guinea pigs, rabbits and mice that received filtered or unfiltered supernatant fluid remained free from symptoms during observation periods of 8, 10 and 14 days, respectively. On the 6th day after inoculation blind passage by intracerebral inoculation of the supernatant

fluid from suspensions of mouse brain, prepared from both groups of inoculated mice by grinding the brains in Simms' salt solution,[6] again failed to elicit signs of illness during 10 days' observation. Attempts by these methods to demonstrate a virus or other infective agent apart from the bacteria in the exudates thus resulted negatively.

3. Infectivity of mixed cultures

Procedure.—Two sets of serial cultures of M.P. exudate were prepared in the following way. In one set, 3 small deep plates of Proske-Sayers[7] AFK medium[3] as used for primary cultivation of spirochetes were inoculated with one drop in the well or by stab, or by both drop and stab. In the second set, 3 sheep blood agar plates were inoculated with 6 or 8 separated drops ("spots"), which were allowed to dry without being spread before the plates were inverted. The initial plates were inoculated with M.P. exudate after 12 guinea pig passages; for subsequent plates the inoculum was the culture suspension used for animal inoculation, as described below. All cultures were incubated at 37 C for 5 days in Brewer jars made anaerobic with hydrogen and containing 5% carbon dioxide. After incubation one plate of each set was selected and a suspension of the growth prepared in extract broth. The agar from the AFK plates, including all bacterial growth in the well and the spirochete haze to its periphery, was scooped out aseptically with a small spatula and suspended in 2.5 ml of broth with the aid of a glass tissue grinder. Five spots from one blood plate, including about one-half the thickness of the underlying agar, were suspended in 2.5 ml of broth in the same way. After darkfield examination of the suspensions, each was inoculated subcutaneously into 2 guinea pigs, using 1 ml as inoculum. Exudate from 1 guinea pig of each pair was transferred to 2 fresh guinea pigs on the 4th, 5th or 6th day after inoculation, using 0.1 ml subcutaneously. All guinea pigs were examined daily for 7 days. Ten serial cultures were prepared in each medium at 5-day intervals.

Results.—Throughout the 10 serial transfers the cultures in both mediums maintained a characteristic appearance with little variation. In the AFK plates the picture was typical of primary spirochete cultures, with bacterial growth limited to the well and stab, and spirochetes growing as a haze in the surrounding agar.[3] On the blood plate the culture appeared as a round spot of yellowish, rough, flat, mixed surface growth with a peripheral raised border of the same character, surrounded by one or more peripheral fringes of whitish or colorless translucent effuse surface growth. Beyond this was an outermost ring of partial hemolysis. Differential sampling and darkfield examination of these zones showed that the center contained mixed growth, including spirochetes, vibrios and fusiform bacilli, with cocci predominating; the raised border of the spot had a similar composition, with fewer spirochetes and more abundant cocci; a rough, white, lobate fringe next beyond consisted mainly of fusiform bacilli, with other forms present but cocci not numerous; a more peripheral, finely stippled, rough, translucent fringe contained fusiform rods, motile rods and vibrios, with some spirochetes but no cocci; while the outermost layer of surface growth, very finely stippled, effuse and transparent in appearance, when sampled carefully to avoid the underlying agar, consisted mainly of motile small vibrios. The underlying agar in this area contained spirochetes, and in the partially hemolytic zone beyond the surface growth, spirochetes were found free from bacteria.

The suspensions prepared from the two sets of cultures, when examined by darkfield illumination, showed consistent differences from one another, and both were different in appearance from the typical darkfield picture of fusospirochetal exudates. Cocci appeared to be relatively more numerous in both types of culture suspensions than

6. Simms, H. S. and Sanders, M. 1942, Arch. Path. **33**: 619.
7. Proske, H. O. and Sayers, R. R. 1934, U. S. Pub. Health Rep. **49**: 839, 1212.

in exudates, and more numerous in blood agar than in AFK cultures. As compared with exudates, there appeared to be a relative increase in the AFK cultures of small fusiform bacilli and motile vibrios, and in the blood cultures of large motile spirilla. In both types of culture large fusiform rods and long filaments were prominent. Spirochetes were numerous in both types, but relatively less active than in exudates. In general a higher proportion of these forms was motile in the blood with passage exudate. An apparent diminution of infectivity was observed with animals of the 4th blood culture generation and of the 5th and 6th AFK culture generations. The results obtained with subsequent AFK cultures, in which full infectivity seemed to be regained, indicated that this was a chance phenomenon. In the blood culture series the results with later generations were somewhat irregular, and may indicate a significant loss of infectivity. Whereas fatal (++++) fusospirochetal infec-

TABLE 3.—*Infectivity for guinea pigs of ten serial mixed cultures initiated with whole fusospirochetal exudate, series M.P.*

Culture passage	Cultures in AFK spirochete plates				Spot cultures on anaerobic blood agar plates			
	Original animals		Passage animals (from*)		Original animals		Passage animals (from*)	
1	++,	++*	++,	++	++,	++++*	++,	++++
2	++,*	+++	++++,	++++	++,*	++	++,	++++
3	++,*	++++	++,	++	++,*	++	++,	++
4	++,*	++	+++,	+++	+,*	+	+,	±
5	++,*	++	+,	±	+,	++*	+++,	±
6	+,*	+	+,	+++	++,	+*	±,	±
7	+++,	++++	+,	±	+,	+++*	+,	+
8	++,*	+	+++,	+++	+§			
9	++,*	++	++++,	++++	+,	++*	+,	+
10	++++,	++*	++,	+++	++,‖	++‖		

Inoculum, 1 ml of a suspension of mixed culture with agar in extract broth, subcutaneously.
Notations for lesions in animals follow the key given under table 1.
§ The volume of culture suspension available in this instance was sufficient for inoculation of only one animal. The lesion evacuated and drained on the 6th day, precluding passage.
‖ In both animals, draining of the lesion on the 6th day precluded passage.

agar suspensions than in those prepared from AFK plates.

The results obtained in guinea pigs inoculated with these culture suspensions are summarized in table 3. The recording scheme is the same as that used in the previous experiments in this report. The lesions that followed inoculation of 1 ml of culture suspension were in general fully equivalent in degree and in kind to those produced with 0.1 ml of passage exudate, and transfer of exudate from the culture-inoculated animals yielded typical fusospirochetal responses with few exceptions. Exudates from both primary and passage animals, when examined under darkfield illumination, showed a picture indistinguishable from that of animals inoculated tions were produced throughout the AFK series, either in the culture-inoculated animals or in the passage animals, no fatalities appeared in the blood culture series beyond the second culture generation. It is nevertheless not clear that the guinea pigs inoculated with 10th generation blood culture suspensions were less affected than the primary animals of the 2nd or 3rd generations. Unfortunately, the lesions in both 10th generation animals had begun to drain before passage was attempted. Draining lesions have been avoided for passage because of changes in the exudate flora associated with healing, and the possibility of introducing extraneous microorganisms into the passage flora.

4. Isolation and study of additional pure cultures

Procedure.—From one of the AFK plates of the first mixed culture generation, two bacteria-free cultures of spirochetes were grown in Noguchi tube cultures of the AFK medium. These were designated MPAI (from an inner growth haze) and MPAO (from an outer haze), respectively. They are described further in another report.[3]

Portions of the mixed culture suspensions in the 3rd, 5th and 6th culture generations, in both blood agar and AFK series, were subcultured as follows. Each suspension was streaked serially on 2 sheep blood agar plates[1] and on 2 plates of the Proske-Sayers[7] medium as used for spirochetes,[3] but without fresh tissue (AF). A third blood plate was streaked separately, as were 2 plates of the AF medium to which 0.003% of gentian violet had been added. All plates were incubated in anaerobic jars with hydrogen and 5% carbon dioxide at 37 C. One of each pair of plates was examined and subcultured after 3 days' incubation, the other after 5 days. The extra blood plate was incubated for 8 days. Selected colonies, identified as to colony form and by means of gram-stained and darkfield preparations, were isolated in broth either directly or after restreaking on sheep blood agar. The broth mediums used were Bacto Brewer thioglycollate medium (No. B36, here designated BX), or variants of this medium, all prepared without agar, containing no additions (BF), 0.1% cysteine hydrochloride (BFC), and 0.1% cysteine hydrochloride plus 10% ascitic fluid (BFCAF).

Thirty-nine pure cultures of bacterial forms other than spirochetes were isolated by these means, and their first pure subculture stored in solid carbon dioxide. Since it was apparent that these included replicates both of the 21 strains previously isolated from M.P. exudates[3] and within the new group, although distinctive forms were evidently present as well, all 60 cultures were screened by means of a series of morphological and biochemical tests. Streaked sheep blood agar plates were incubated aerobically to test for aerobic growth. Anaerobic cultures were prepared on sheep blood agar, in BX broth, in the sugar-free B.B.L. thioglycollate broth (No. 136, here designated BBL), and in the BBL broth with the addition, respectively, of 1% of dextrose, sucrose and mannite. Shake tube cultures were prepared in the BX medium containing 1% agar, and in a medium consisting of equal parts of (a) Bacto veal infusion with 1% agar and (b) peptone iron agar. The shake cultures were incubated aerobically. All inoculations were made by capillary pipette, using 1 to 10 drops, depending on the turbidity of the seed culture. The cultures were incubated at 37 C for the following periods: streptococci, 3 days; fusiform rods and nonmotile gram-positive or gram-negative rods, 4 days; and motile rods and vibrios, 5 days. Colony form was studied on the blood agar cultures, and both gram stains and darkfield preparations were made from the colonies and from the BX broth cultures. The latter were also used for indol tests. The four BBL mediums were tested for pH with a glass electrode potentiometer. The BX agar shake cultures were observed for characteristic appearance of growth and gas production, and the veal infusion-peptone iron agar shake cultures for hydrogen sulfide production.

Of the 60 cultures thus tested, 7 were eliminated (all non-motile rods), 5 of these having failed to grow in any of the test mediums and 2 having been contaminated. Comparison of the data for the remaining 53 cultures indicated that 24 more could be eliminated as replicates, leaving 29 cultures that seemed distinctive in one or more of their characteristics. This group was rechecked by the morphological and biochemical tests previously used, and was tested in addition for reduction of nitrate, production of ammonia and formation of urease, using BBL medium with appropriate modifications. For reduction of nitrate, sodium nitrate was added to a final concentration of 0.1%. After adequate incubation the presence of nitrite was determined by suitable adaptation of the method of Bratton and Marshall.[8] Ammonia production was determined by making the culture alkaline with sodium hydroxide and warming with a strip of moist phenol red paper suspended in the tube. A prompt intense color change was interpreted as a positive test. The urease test was performed by adding urea to a concentration of 0.2% after maximal growth, incubating 5 additional hours at 37 C and testing for ammonia with phenol red paper but without alkalinization. It was previously determined that this procedure could detect 2×10^{-6} g of urease (from Jack bean meal) in 10 ml of medium. Blanks on sterile medium were performed for all tests.

Results.—Of the 5 cultures of spirochetes isolated from the M.P. exudate strain, the two newer cultures, MPAO and MPAI, were presumed to duplicate the three others. In fact, since MPAI,

8. Bratton, A. C. and Marshall, E. K., Jr. 1939, J. Biol. Chem. **128:** 537.

having been derived from the inner haze in an AFK spirochete plate, was regarded as including MPAO, which came from the outer haze on the same plate, the former may have included all the M.P. spirochetes. In the absence of means sufficiently refined to test these hypotheses it is not known whether all the separated cultures were or were not variants of a single species.

Identifying data for the remaining 29 cultures are given in table 4, in which 5 morphological groups are recognized: (a) fusiform rods; (b) motile rods; (c) nonmotile gram-negative rods; (d) nonmotile gram-positive rods; and (e) streptococci.

Of the *fusiform rods*, the first 4 cultures listed in table 4 (F39a, F44, F54 and F55) may be identical, since they showed only minor morphological and small quantitative biochemical differences. These strains correspond in their characteristics with the *Fusobacterium* of Bøe.[9] Strain F48 may also belong in this group but was morphologically atypical and failed in both trials to produce gas in BX shake cultures. Strains F41 and F47 seemed distinctive. In comparison with the other fusiform rods, these two strains were distinctly poorer growers, and neither fermented dextrose nor formed indol. The difference between the two was principally morphological, and they may have represented rough and smooth variants of the same species. It may be noted that all 7 of these cultures were gram-negative, and none was motile.

The *motile rods* seem to fall into 3 distinct groups. Strain VB7a appeared to form a class apart. As noted previously[2] this culture was derived from an AFK spirochete plate and, alone among the M.P. cultures other than spirochetes, failed consistently to grow on the surface of solid media either aerobically or anaerobically. Its motility was distinctively that of the vibrios of exudates: active and "tumbling," i.e., showing erratic rotation around the short axis. As shown in table 4, it grew moderately well in fluid mediums and formed acid from dextrose and sucrose with an average terminal pH of 5.2 in the former. It did not form indol or hydrogen sulfide. Growth occurred in the sugar-free BBL medium in the first trial but not in the second; hence the tests for reduction of nitrate and for ammonia and urease production were unsuccessful. The second group of motile rods comprised strains VB9, VB11 and B22. These were small gram-negative rods, with distinctive but perhaps minor morphological differences among the three cultures. Their motility was more sluggish than that of strain VB7a and did not show tumbling. Biochemically they were consistent nonfermenters. None formed indol or hydrogen sulfide, and all produced ammonia and urease. Strain VB10 may correspond with the *Spirillum sputigenum* of Miller.[10] It is a distinctly larger form than the others, with a marked tendency to form thick spirals as well as comma-shaped elements. It grew more abundantly than the smaller forms, fermented mannite as well as dextrose and sucrose, produced ammonia and gave a weak urease reaction.

The *nonmotile gram-negative rods* showed among themselves differences that were principally morphological and quantitative. They differed in cell morphology and in colony form, and two of them (B23 and B28) produced greening of blood agar after exposure of anaerobic plates to air. This group may intergrade with the nonmotile gram-positive rods,

9. Bøe, J. 1941, Norske Videnskaps-Akademi I Oslo I. Mat.-Naturv. Klasse, No. 9.

10. Miller, W. D. 1890, The Microorganisms of the Human Mouth, Philadelphia, S. S. White Co.

TABLE 4.—*Characteristics of pure cultures, other than spirochetes, isolated from fusospirochetal exudate (strain M.P.).*

Culture	Colony form	Cell morphology	Gram	BBL† Growth in BX*	BBL† Growth	BBL† pH	Fermentation in BBL† Dextrose	Sucrose	Mannite	pH in dextrose	Indol	Hydrogen sulfide	Nitrate reduction	Ammonia	Urease	BX* shake cultures Growth	Gas
F39a	Smooth, flecked	Fusobacterium	0	4+	3+	6.7	A	0	0	6.0	+	±	0	+	+	3+	V
F44	Smooth	Fusobacterium	0	4+	3+	6.7	A	0	0	6.0	+	±	0	+	+	2+	V
F54	Rough	Fusobacterium	0	3+	3+	6.7	A	0	0	6.2	+	+		+	+	2+	V
F55	Rough	Fusobacterium	0	3+	3+	6.7	A	0	0	6.2	+	+	0	+	+	3+	V
F41	Tiny, smooth	Filaments	0	±	±	6.9	0	0	0	6.8	0	−	−	±	0	V	0
F47	"Cocklebur"	Short blunt fusiform rods	0	+	+	6.7	0	0	0	6.6	0	±	−	+	+	V	0
F48	Smooth to rough	Variable blunt fusiform rods	0	4+	3+	6.8	A	0	0	6.0	+	±	0	+	+	2+	0
VB7a	None	Small granular curved vibrios, motile	0	2+	2+	6.9	A	A	0	5.2	0	0	−	−	−	2+	0
VB9	Smooth, umbonate	Slender curved rods, motile	0	±	+	7.0	0	0	0	6.7	0	0	−	+	+	0	
VB11	Effuse, rhizoid	Small ovoid rods, motile	0	+	±	7.0	0	0	0	6.7	0	0	0	+	+	0	
B22	Smooth, umbonate	Slender straight rods, motile	0	+	+	6.9	0	0	0	6.6	0	0	0	+	+	0	
VB10	Smooth to matt	Large thick double-curved rods, motile	0	3+	+	6.8	A	A	A	5.4	0	0	0	+	±	3+	0
B21	Smooth	Small rods	0	+	±	7.0	0	0	0	6.6	−	−	−	+	0	0	
B23	Variable, greening	Slender rods, some curved	0	±	+	7.0	0	0	0	6.7	0	0	0	+	+	+	0
B28	Rough, greening	Slender rods, some curved	0	±	2+	7.1	0	0	0	6.8	0	0	−	+	+	0	
B30	Rough	Small ovoid rods	0	±	+	7.1	0	0	0	6.8	0	0	−	+	+	0	
R39	Smooth	Variable small rods	0	2+	±	7.0	0	0	0	6.7	0	0	−	+	±	3+	0
R26	Smooth	Variable small rods	±	2+	±	7.0	0	0	0	6.7	0	0	−	±	0	2+	0
R27	Smooth	Variable small rods	±	+	±	7.0	0	0	0	6.8	0	0	−	±	0	2+	0
R29	Smooth	Small rods	+	±	+	7.1	0	0	0	6.8	0	0	−	+	+	±	0
R43	Smooth	Small rods	±	+	+	7.1	0	0	0	6.9	0	0	−	+	+	+	0
B29	Smooth	Small ovoid rods	±	±	2+	7.2	0	0	0	6.9	0	−	−	+	+	+	0
R28	Smooth, opaque	Thin bent rods	+	3+	2+	7.0	A±	0	0	6.4	0	0	0	±	±	2+	+
R40	Smooth, opaque	Short ovoid rods	+	4+	2+	7.0	A	0	0	4.9	0	+	0	0	0	4+	0
R41	Smooth, opaque mucoid	Short plump rods	+	3+	2+	7.0	A	0	A	5.1	+	0	+	+	+	4+	0
S24	Matt, greening‡	Streptococci	+	4+	+	6.9	A	A	0	4.6	0	0	0	+	±	2+	0
S26	Smooth	Large diplococci, streptococci	+	+	2+	6.6	A	A	A	5.6	0	0	0	0	0	2+	0
S27	Smooth	Small diplococci, streptococci	+	2+	3+	6.8	0	0	0	6.6	0	+	0	+	+	3+	0
V11	Smooth	Small diplococci, streptococci	+	2+	2+	6.9	0	0	0	6.7	0	+	0	+	+	2+	0

* BX, Bacto Brewer thioglycollate medium No. B36.
† BBL, Baltimore Biol. Labs. thioglycollate medium No. 136 (sugar-free).
‡ This strain alone among those listed showed growth aerobically on blood agar.
A, acid; 0, negative, +, positive; ±, weak positive or doubtful; −, no growth in the test medium; V, variable.

several of which retained the gram stain weakly or irregularly. Strain R39, in fact, was recorded as gram-positive when first isolated, while strain B29 was placed originally in the gram-negative group. These reactions were found to be reversed for the 2 strains in the screening tests. A culture interval of less than 4 days might have given different results. It may be noted from the data in table 4 that strains R39 and R26 were identical in all but two characteristics, gram reaction and urease production.

The *nonmotile gram-positive rods*, at all events, appear to be rather heterogeneous. The first 5 strains listed (R26, R27, R29, R43 and B29) were similar to one another and also to the group of gram-negative rods. The remaining 3 strains all showed more vigorous growth. Strain R28, described previously,[2] is distinctive both morphologically, producing characteristically bent but unbranched rods, and biochemically, being a weak fermenter of dextrose only. Strains R40 and R41 were both strong acid producers from dextrose, the latter fermenting mannite as well. The two differed from one another and from the other members of the group with respect to production of indol, hydrogen sulfide, ammonia and urease and in reduction of nitrate.

Among the *streptococci*, strain S24 was a typical α streptococcus, and was the only member of the entire group to grow aerobically on blood agar plates. Its reactions appear to place it in the *Streptococcus mitis* group of Sherman, Niven and Smiley.[11] The other 3 strains were anaerobic. Strain S26 was distinctive in showing larger cells than the others, in fermenting all 3 test carbohydrates, and in giving negative results with the other biochemical tests. Strains S27 and V11 may have been identical, being similar in morphology, nonfermentive, producing hydrogen sulfide, ammonia and urease.

11. Sherman, J. M., Niven, C. F. and Smiley, K. L. 1943, J. Bact. **45**: 249.

5. *Pathogenicity of recombined culture mixtures*

Procedure.—The individual strains were transferred from dry ice as pure cultures according to a prearranged schedule so that seed cultures became available on a single day. Cultures of spirochetes were transferred twice in deep tubes of AFK medium in duplicate, incubated 6 and 3 days, respectively, at 37 C. The other cultures were transferred only once to yield seed cultures. They were divided into three groups based on incubation time at 37 C: (a) the 4 streptococcus cultures, 3 days; (b) the 5 motile rods and strains R29, B23 and F41, 5 days; and (c) the 17 remaining cultures, 4 days. The 29 cultures were all inoculated in duplicate into BX and BF broths, using 8 to 10 drops of inoculum, and on blood agar slants, using 1 or 2 drops. These were incubated anaerobically. All were also streaked on blood agar plates and incubated aerobically. The aerobic cultures, examined a day earlier than their anaerobic counterparts, were all negative, including strain S24, which grew heavily under anaerobic conditions.

Table 5 indicates the composition of the 12 recombination groups. Group A1 included all 34 cultures. In groups A2 to A6, the grouping was based on omission of the aerobic streptococcus, S24 (A2), the nonmotile gram-positive rods (A3), the nonmotile gram-negative rods (A4), both latter groups (A5), and all the nonmotile rods other than fusiforms together with the aerobic streptococcus (A6). It may be noted that at this time strain R26 was classed among the gram-negative rods. In the groups B1 to B6, a similar order was based on 17 strains that appeared to comprise the more distinctive cultures. As indicated at the bottom of table 5, the 12 mixtures thus included from 9 to all 34 cultures.

On the day of harvesting of the seed cultures, the spirochete strains were emulsified in extract broth in glass tissue grinders, using a pool of all 5 strains for the A series of recombination groups. Of the remaining strains, that culture in one of the three mediums which showed most abundant growth was selected as seed. All cultures grew well in at least one tube except strain F41, which showed scanty growth and grew best on the blood agar slants. The selected broth cultures were concentrated by centrifugation. Twelve mixtures were prepared to contain the cultures

TABLE 5.—*Culture combinations as prepared for inoculation of guinea pigs.*

Culture	Culture combination group											
	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
TM13	+	+	+	+	+	+						
TM13b	+	+	+	+	+	+						
TM13d	+	+	+	+	+	+						
MPAO	+	+	+	+	+	+						
MPAI	+	+	+	+	+	+	+	+	+	+	+	+
F39a	+	+	+	+	+	+	+	+	+	+	+	+
F44	+	+	+	+	+	+						
F54	+	+	+	+	+	+						
F55	+	+	+	+	+	+						
F47	+	+	+	+	+	+	+	+	+	+	+	+
F48	+	+	+	+	+	+						
F41	+	+	+	+	+	+	+	+	+	+	+	+
VB7a	+	+	+	+	+	+	+	+	+	+	+	+
VB9	+	+	+	+	+	+						
VB11	+	+	+	+	+	+	+	+	+	+	+	+
B22	+	+	+	+	+	+						
VB10	+	+	+	+	+	+	+	+	+	+	+	+
B21	+	+	+				+	+	+			
B23	+	+	+									
B28	+	+	+				+	+	+			
B30	+	+	+									
R29	+	+	+									
R26	+	+	+									
R27	+	+		+			+	+		+		
R29	+	+		+								
R43	+	+		+			+	+		+		
B29	+	+		+			+	+		+		
R28	+	+		+			+	+		+		
R41	+	+		+								
R40	+	+		+			+	+		+		
S24	+		+	+	+		+		+	+	+	
S26	+	+	+	+	+	+	+	+	+	+	+	+
S27	+	+	+	+	+	+						
V11	+	+	+	+	+	+	+	+	+	+	+	+
Totals	34	33	27	28	21	20	17	16	12	15	10	9

other than spirochetes as listed in table 5 by pooling 1 drop by capillary pipette from the BX culture sediments or from the lighter BF sediments or slants, or one 2.5 mm loopful of the heavier BF sediments or slants, using either the surface growth or the water of condensation of the slants.

Mixed cultures were then prepared in an effort to duplicate those inoculated with whole M.P. exudate, as described earlier in this paper. AFK spirochete plates and blood agar plates were inoculated in triplicate with each recombination mixture except for group A1, in which 6 plates of each kind were used. The AFK plates were inoculated first with a drop of spirochete emulsion in the well, followed by a drop of appropriate bacterial mixture, using capillary pipettes. A stab was then made with a sterilized needle through the base of the well, short of the floor of the plate. The blood plates were inoculated as spot cultures with a drop of spirochete emulsion, followed in the same spots with a drop of bacterial mixture for each. Each plate received 8 such spot inoculums. The plates were incubated anaerobically for 5 days at 37 C and studied by the method given above for mixed cultures prepared with whole exudate, including the preparation of culture emulsions, the inoculation of guinea pigs, and the transfer of emulsions to fresh medium. Agar scooped from AFK plates, or 5 spots from one blood plate, were emulsified in 2.5 ml of extract broth. Two plates of each kind were used for group A1, and 4 guinea pigs inoculated with each emulsion. One plate of each was used for the other groups, and each emulsion inoculated into 2 guinea pigs. The guinea pig inoculum was 1 ml subcutaneously. Two or 3 successive subcultures were prepared in each instance, but only the first subculture was tested in guinea pigs. All promising guinea pig exudates were transferred to fresh guinea pigs, using 0.1 to 0.5 ml of exudate subcutaneously.

Results.—The first set of mixed cultures in both mediums showed typical mixed bacterial growth but little or no evidence of spirochetal outgrowths. Both grossly and in darkfield preparations, the B series showed slightly greater growth of spirochetes than the A series, and the AFK plates were supe-

rior in this respect to the blood plates. Vibrios or other motile rods were not seen in any of the emulsions. The second set of mixed cultures, inoculated with emulsions of the first, yielded distinctly more abundant growth of spirochetes both grossly and microscopically in 17 of the 24 recombination groups. In 6 of these (AFK cultures from AFK emulsions, groups A6, B2 and B3; blood agar cultures from AFK emulsions, groups A1, A2; and blood agar culture from blood agar emulsion, group A2), the appearance of the cultures closely resembled the mixed cultures prepared with exudate. Under the darkfield microscope emulsions of these 6 cultures revealed an apparently complete flora, but spirochetes and motile bacilli and vibrios were distinctly less numerous than in exudates or exudate cultures. A third set of cultures inoculated from the second proved generally poorer than its predecessor in spirochetes and vibrios; and a fourth set, inoculated from the third, gave similar or still poorer yields.

Guinea pigs inoculated with emulsions from the first two sets of cultures, and passage animals that received exudates from the primary guinea pigs, in many instances showed lesions more nearly approximating the characteristic fusospirochetal response than any previously inoculated in these studies with recombined cultures;[1,2] but none was completely characteristic. Large (++) lesions appeared in 5 of the recombination groups (A2, A3, A6, B1, B3), and spreading but nonfatal (+++) lesions were found in 6 (A3, A5, B1, B2, B3, B5). There were no fatal lesions. Exudates aspirated from these lesions were obviously less abundant than in typical fusospirochetal lesions and were either odorless or nearly so, the odor when present being faint and atypical. The responses in animals inoculated from the second set of cultures were only slightly superior to those of the first set, while passage animals in both sets responded on the whole less characteristically than those that received culture emulsions. Darkfield examination of the exudates revealed few or no spirochetes in most instances. Motile vibrios appeared, accompanied by more abundant spirochetes, in only one group, B4, in both animals inoculated with the second set of blood agar cultures. These exudate findings were associated with very small local abscesses. On passage these exudates gave diminished responses.

The results are summarized in table 6, which presents comparative darkfield findings for the individual cultures and both gross and darkfield findings for individual primary and passage animals. Examination of the data shows no apparent relationship between the character of the findings and the particular culture combination used. No parallelism is evident between the A and the corresponding B groups, except perhaps in A2 and B2. The former with both AFK and blood agar cultures of the second set, gave the most consistent responses, while in group B2 (second AFK set) appeared the only animal to show a large spreading lesion with fairly abundant spirochetes. It may be noted that in these two groups the principal culture omitted from the combination was the aerobic streptococcus, strain S24. Comparison of the data for groups B4 and B5 exemplifies the apparently random character of the findings. Darkfield findings in both cultures and animal exudates in group B4 were among the most nearly typical of fusospirochetal infection; but these were associated with only small or moderate lesions. In group B5, two animals of the blood agar series showed large spreading lesions, but no spirochetes were found in their exudates. The two groups differed only in that the B4 culture

TABLE 6.—*Results of inoculation of recombined cultures into guinea pigs.*

	Serial cultures	*A* series, guinea pigs		Serial cultures	*B* series, guinea pigs
A1	AFK(0) B(+++) B(+) AFK(+)	+(±); ±(±); +(0); +(0) ±(0); ±(0)	B1	AFK(±) AFK(+) B(+)	+++(±); ±(−) +++(+)T = ±(0), ±(−); +(+)T = ±(±), ±(±)
A1	B(±) B(±) B(+) AFK(+)	+(0)T = ±(−); ±(0); ±(0); ±(0) ±(±)T = +(0)	B1	B(0) B(0) B(0)	+++(0); ±(−) ±(0)
A2	AFK(0) B(+++) B(+)	++(0); ++(0) +(+)T = ±(±), ±(±); ±(+)T = +(±), ±(±)	B2	AFK(+) AFK(+++) B(+)	±(0)T = ±(−), ±(−); +(+)T = ±(−) +++(0); +++(+)T = ±(0)
A2	B(0) B(+++) B(+) AFK(+)	+(0); +(+)T = ±(±) +(+)T = +(±), ±(+), ±(+); ±(0)	B2	B(0) B(0) B(0)	+++(0); +(−) ±(−); +++(0)
A3	AFK(±) B(+) B(+)	+(0); ++(0)T = ±(−), ±(−) +(0); +++(0)	B3	AFK(+) AFK(+++) B(+)	+(0); +(−) ±(0)T = ±(0); +(±) T = ±(0)
A3	B(0) B(±) B(0)	±(0); ±(−) ±(±); ±(0)	B3	B(0) B(±) B(+)	+++(0); ++(−) +(0)T = +(0), ±(0); +++(0)
A4	AFK(0) B(+) B(+)	±(0); ±(0) ±(±); +(0)	B4	AFK(±) AFK(+) B(+++) AFK(±)	+(+)T = ±(−), ±(−), ±(−); +(−)
A4	B(0) B(0) B(0)	+(0); +(0) ±(0); ±(0)	B4	B(±) B(+) B(+)	±(+)T = ±(−); +(−) ±(++)T = ±(±); ±(++)T = +(±)
A5	AFK(±) B(+) B(+)	+(0); +(0) ±(0)	B5	AFK(±) AFK(±) B(++)	±(0); +(−) +(−); +(0)
A5	B(0) B(0) B(0)	+++(0); +(−) ±(0); +++(0)	B5	B(±) B(+) B(+) AFK(+)	±(0); +++(0) +++(0); ±(0)
A6	AFK(+) AFK(++) B(+)	+(0); ++(0) ±(0); ±(0)	B6	AFK(±) AFK(+) B(+) AFK(+)	+(0); ±(0) ±(0)
A6	B(0) B(0) B(0)	±(0); +(−) ±(0); ±(0)	B6	B(±) B(+) B(+) AFK(+)	±(0); ±(−) ±(0); ±(0)

Inoculum, 1 ml subcutaneously.
Darkfield findings with both cultures and animal exudates are given in parentheses:
(0), no spirochestes.
(±), less than 10 spirochetes per oil-immersion field; no motile vibrios.
(+), 10 or more spirochetes per field; no motile vibrios.
(++), 10 or more spirochetes per field; motile vibrios present.
(−), not examined.
Notations for lesions in animals follow the key given under table 1.
T = transferred, followed by notations for passage animals separated by commas.
Notations for original animals (including transfers and passage animals) are separated by semicolons.
Example: The entry in the second line under the second B4 indicates that the second passage spot culture on blood agar showed 10 or more spirochetes per field without motile vibrios, and yielded very small local lesions in each of two guinea pigs. Each of these showed 10 or more spirochetes and motile vibrios (as well as other organisms) in their exudates. When transferred the first yielded a very small local lesion containing less than 10 spirochetes per field without motile vibrios; the second produced a moderate local lesion with similar darkfield findings.

mixture contained 5 cultures of gram-positive nonmotile rods which were omitted in B5.

DISCUSSION

The findings in the first group of experiments reported indicate that whole fusospirochetal exudate is a comparatively stable infective mixture. Despite some loss of motility of its component microorganisms, its infectivity seemed unimpaired after standing for 24 hours at 4 C or at room temperature, or for at least 6 hours at 37 C. Together with the sustained infectivity of exudate with increasing volumes of inoculum from 0.1 to 1.6 ml, without change in actual dosage, this finding would seem to indicate that failure to obtain typical fusospirochetal responses with recombined cultures in the previous study[2] could not have been due to the time or volume factors.

The lack of evidence of a virus or comparable infective agent in fusospirochetal exudate in the results of the second group of experiments does not of course prove the absence of such an agent, but a presumption that no such agent is present seems tenable in the

absence of data to the contrary. It may be noted that Tilden and Burrill,[12] Steigman and Scott[13] and unpublished data from this laboratory[14] have shown that herpes virus is not an essential component of the fusospirochetal infective complex. The present findings are consistent with the foregoing and embody a further suggestion. Aside from the failure of these experiments to demonstrate a virus directly, the finding that mixed cultures started with exudate retained their infectivity apparently unimpaired through 10 serial transfers adds indirect evidence to the same effect. While the dilution of original inoculum of exudate involved in the ten serial cultures was not measured, an estimate indicates that it must have been many times the dilution required to eliminate the infectivity of such exudates. In a previous report[2] it was shown that the lethality of 0.1 ml of inoculum for guinea pigs was lost at a dilution of 1:16, while infectivity tended to disappear correspondingly in the dilution range 1:32 to 1:128. The dilution involved in the present series of mixed cultures may be estimated conservatively as follows. The original inoculum was 1 drop of exudate, and the growth-laden agar from which the inoculum for subsequent cultures was prepared had a volume of the order of 1 ml. The dilution in the first (AFK) plate was thus at least 1:10, while in subsequent plates it was 1 drop of a suspension of approximately 1 ml of agar culture with 2.5 ml of broth, or at least 1:30. The total dilution was thus at least $1:10\times30^9$, or more than $1:10^{15}$. Since the tenth AFK culture yielded apparently unimpaired infection it seems unlikely that an infective agent incapable of multiplying in the AFK medium, such as a virus, could have taken part in the process.

The complex, mixed population of microorganisms which comprises or includes the pathogenic principle of fusospirochetal exudates is evidently able to survive through at least 10 serial subcultures in artificial mediums, and was apparently unaffected by the observed changes, different for the two mediums used, in either the proportions of the different microorganisms, or the morphology of some of them, or both. These changes seemed to be reversible adaptations to the differing environments of culture mediums or guinea pig tissues, since exudates from guinea pigs inoculated with culture emulsions at each step in the series showed an immediate reversion to the pattern of proportion and morphology typical of exudates. Preservation of the pathogenic principle under these conditions, and its loss or serious modification under the conditions of the next set of experiments, suggests a highly complex and delicate ecological balance in a mixed microbic population which is maintained in association but lost in the effort to separate and recombine the elements of the population. This, admittedly, is by no means the only possible explanation for the findings in the last set of experiments.

When individual strains of microorganisms were separated from the foregoing mixtures and added to cultures previously isolated from M.P. exudates, a comparison of their differential features showed at least 17 and perhaps as many as 34 different cultures. Although the group seemed to include the whole gamut of morphological varieties seen in exudates, it has not been demonstrated whether one or more essential species remained uncultivated. When these cultures were recom-

12. Tilden, E. B. and Burrill, D. Y. 1947, Northwestern Univ. Bull. **47**: 3.
13. Steigman, A. J. and Scott, T. F. M. 1947, Proc. Soc. Exper. Biol. & Med. **64**: 244.
14. Clark, A. R. 1944, Unpublished data.

bined in an effort to reduplicate infective mixed cultures, they failed to yield the completely typical pattern either in the mixed cultures or in the guinea pigs inoculated from the cultures. The guinea pig responses were more nearly typical than those obtained in earlier experiments in this laboratory. The lesions tended to be larger than formerly, and in some instances there was a characteristic extension of the infection through the subcutaneous tissues. Darkfield examination showed in some instances a flora that seemed qualitatively complete, but the concentration of spirochetes and motile bacteria was never so high as in typical fusospirochetal lesions. The exudates, moreover, were less abundant and distinctly less foul than is characteristic, and they were clearly lacking in infectivity, since passage guinea pigs showed generally poorer responses than the primary animals.

These findings would seem to call into question the reports by previous workers[7,15–17] of the production of typical fusospirochetal lesions with recombined cultures. The analysis of these reports given in a previous paper in this series[1] indicates that they left certain doubts unresolved. It is suggested that the precise nature of fusospirochetal infection remains to be determined, and that a reproducible procedure for duplicating it with identifiable cultures continues to be a subject for research.

SUMMARY

Further studies are reported on a "strain" (designated M.P.) of fusospirochetal infection maintained by guinea pig passage of lesion exudates. The infectivity of such exudates for guinea pigs was found apparently unimpaired after storage for 24 hours at room temperature or at 4 C, and for at least 6 hours at 37 C, despite partial loss of spirochetal motility. No evidence of loss of infectivity resulted from increasing the volume of inoculum of exudate by dilution in extract broth in the range 0.1 to 1.6 ml, the actual dose remaining constant.

Attempts to demonstrate a virus or similar infective agent in fusospirochetal exudate by filtration or centrifugation and inoculation of the filtrate or supernatant fluid subcutaneously into guinea pigs, on the scarified cornea of rabbits, or intracerebrally into mice gave negative results.

The infectivity of mixed cultures in two mediums, initiated with M.P. exudate and maintained by subinoculation through 10 serial culture transfers, showed no appreciable impairment with one medium and questionable impairment with the other. Changes in morphology of some of the contained microorganisms, or in the proportions of them, or both, were characteristic of the cultures but reverted to the typical exudate pattern in the guinea pigs inoculated at each passage. It is pointed out that these findings strengthen the view that a virus is not an essential component of the fusospirochetal infective complex.

From the mixed cultures, 39 new strains of bacteria were isolated and studied with 21 cultures previously derived from the M.P. series. Screening by morphological and biochemical criteria reduced these 60 cultures to 29, and a further elimination of apparently related forms left 16 seemingly distinctive cultures, including fusiform rods, motile rods or vibrios, nonmotile gram-positive and gram-negative rods

15. Kritchewski, B. and Seguin, P. 1920, Rev. de stomatol. **22**: 41.
16. Smith, D. T. 1930, J. Infect. Dis. **46**: 303.
17. Smith, D. T. 1932, Oral Spirochetes and Related Organisms in Fuso-Spirochetal Disease, Baltimore, Williams & Wilkins Co.

and streptococci. Except for one greening streptococcus, all cultures failed to grow aerobically. Recombination of these 29 cultures together with 5 cultures of spirochetes from the M.P. series, in groups of from 9 to all 34 cultures, using the mixed culture procedure found to be successful when initiated with whole exudate, yielded both cultures and guinea pig responses that fell short of being fully characteristic of fusospirochetal infection.

THE EFFECT OF ANTIBIOTICS ON AGENTS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP

I. THE EFFECT OF PENICILLIN

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The agents of the psittacosis-lymphogranuloma group resemble bacteria in their susceptibility to sulfonamides and antibiotics. In addition, recent evidence indicates that in many respects they are affected by the drugs as are the bacteria. Morgan (1948a and b) has shown that the chemotherapeutic action of sulfadiazine on psittacosis virus is antagonized competitively by *p*-aminobenzoic acid and non-competitively by pteroylglutamic acid. He has suggested that, as in the case of bacteria, pteroylglutamic acid is a required vitamin for the growth of psittacosis virus. Huang and Eaton (1949) also found that *p*-aminobenzoic acid reverses the action of minimal therapeutic doses of sulfonamides in mice infected by the intranasal route with the viruses of mouse pneumonitis or lymphogranuloma venereum. Furthermore, Loosli (1949) has observed that penicillin induces plaque formation in both intra- and extracellular mouse pneumonitis vesicles. This type of abnormal growth is reminiscent of the effect of penicillin on bacteria (Pratt and Dufrenoy, 1947).

The agents of the psittacosis-lymphogranuloma group offer a unique opportunity to elucidate some of the potentialities and limitations of viral chemotherapy and, with the help of chemotherapy, the nature of host-viral relationship. It is true that the doubt has arisen that antimetabolites may prove altogether ineffective against parasites devoid of independent metabolism, and the general tendency has been to exclude the psittacosis-lymphogranuloma group from other viruses (Rake, 1948). But this doubt is not entirely justified. It is possible that the sulfonamides and antibiotics cause subtle alterations in host metabolism and thus indirectly interfere with viral growth. In fact, a close dependence of psittacosis virus multiplication on host metabolism has clearly been shown by Yanamura and Meyer (1941) and of feline pneumonitis by Moulder and Weiss (1951). Also, several attempts have failed to demonstrate a respiratory activity of separated feline pneumonitis elementary bodies (Moulder and Weiss, 1951), comparable to that of rickettsiae (Bovarnick and Snyder, 1949). Thus, the agents of the psittacosis-lymphogranuloma group, even though larger than some bacteria and capable of developing extracellularly in the alveoli of mouse lungs (Weiss, 1949), appear to conform to the characteristics of viruses in their host-parasite relationship.

This study has been undertaken in an effort to understand the effect of antibiotics on the general biology of viruses, their relationship to host cells, and the mechanism which makes one drug superior to another. It began with a study

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of penicillin and was extended to a more efficacious drug, aureomycin (Gogolak and Weiss, 1950).

The effectiveness of penicillin against agents of the psittacosis-lymphogranuloma group has been adequately demonstrated in experimental infections as well as in clinical trials. The results have recently been summarized by Hamre and Rake (1947), Eaton et al (1948), and Goggio (1949). In general, penicillin although efficacious, is not entirely satisfactory.

The agents of feline and murine pneumonitis have been chosen as most suitable for this type of study.

MATERIALS AND METHODS

The egg-adapted strains of feline and murine pneumonitis were the same as those used previously (Weiss, 1949 and 1950). They killed chick embryos by the yolk sac route in dilutions of 10^{-7} and 10^{-4}, respectively, but their pathogenicity for mice was low. The method of preparing virus pools was described in an earlier paper (Weiss, 1950).

The potassium salt of penicillin G produced by Eli Lilly and Company was used throughout. It was obtained in powdered form in ampoules of 100,000 units (approximately 60 mg). Shortly before use, penicillin was dissolved in veal infusion broth and diluted, as needed, in the same medium. The dilution was so gauged that the desired number of units was contained in 0.25 ml of fluid. Any penicillin not used the same day was discarded.

Six-day-old White Leghorn chick embryos were used. Groups of 10 to 30 were used to determine the efficacy of penicillin under each experimental condition. The virus as well as the antibiotic was injected into the yolk sac. The eggs were candled daily and the time of death of the embryos determined. The average day of death (ADD) was calculated as described by Golub (1948). The yolk sacs of representative numbers of dying embryos were Gram-stained and examined for the presence of elementary bodies and the absence of bacterial contamination.

To study changes occurring in the virus structures, enough feline pneumonitis virus was given to kill the embryos in 5 days. Penicillin was given 4 days later (1000 units per embryo), and the yolk sacs of two embryos were collected at each of the following intervals: $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 4, 7, 12, 18, and 24 hours. In addition, yolk sacs of untreated embryos were collected at daily intervals. Essentially the same procedure was used for murine pneumonitis virus. Sufficient virus was given to kill the embryos in 4 to 6 days. Penicillin (100 units per embryo) was administered 3 days after inoculation of the virus, and the yolk sacs of 2 embryos were collected at each of the time intervals indicated above and at 36 hours and daily thereafter up to 10 days. Specimens of untreated embryos were also taken at daily intervals. The yolk sacs were fixed in Zenker-formol for 2 to 4 hours, embedded in celloidin, sectioned, and stained with hematoxylin-eosine-azure II.

RESULTS

1. Effect of varying concentrations of penicillin.—As previously observed (Weiss, 1950), the agent of feline pneumonitis is more virulent for chick embryos than the agent of murine pneumonitis. This is shown in figure 1. Each concentration of feline pneumonitis virus kills the embryos in a shorter time than the corresponding concentrations of murine pneumonitis. Embryos inoculated with the same amount of feline pneumonitis virus die within a short time of each other, and the effect of penicillin on this agent is easily determined. On the other hand, a same inoculum of murine pneumonitis virus kills embryos in a more variable length of time, and the effect of penicillin is gauged with greater difficulty.

A group of embryos were each inoculated with 30,000 LD_{50} (10^{-3} dilution) of feline pneumonitis virus and another group with 3000 LD_{50} (10^{-2} dilution) of murine pneumonitis. Concentrations of penicillin varying from 10 to 10,000 units were given two days later. As shown in figure 1, the antibiotic delayed the average day of death (ADD) of the infected embryos for a length of time dependent on the amount given. With the agent of feline pneumonitis, a progressive delay in ADD was obtained with logarithmic increases of penicillin. With the agent of murine pneumonitis,

this relationship between amount of penicillin and ADD, although similar, was not as clearly seen because the virus is less virulent for chick embryos.

The eventual death of embryos protected with small or large amounts of penicillin was shown to be due to viral infection. Smears from the yolk sacs revealed numerous elementary bodies, and the titer of virus was found to be identical to that of untre..ted embryos.

2. Effect of penicillin on varying concentrations of virus.—Figure 2 illustrates the effect of the same amount of penicillin (1000 units) on varying concentrations (3000 to 300,000 LD_{50}) of feline pneumonitis virus. Similar results were obtained when other doses of penicillin were used (10, 100, and 10,000 units). The antibiotic was administered 2 days after virus inoculation, and therefore, the factor of delayed administration of antibiotic was also present. Embryos receiving 3000 or 30,000 LD_{50} (A and B) were protected as in the previous experiment (fig. 1), except that about 20% of the embryos in B died as soon as the controls. When 300,000 LD_{50} were inoculated, 90% of the embryos died as soon as the controls, but the few surviving embryos lived as long as embryos in the other two groups. These results are similar to those obtained by Hamre and Rake (1947) and suggest that, within limits, the amount of virus present in the embryos did not change the effect of penicillin. But, as better illustrated in succeeding experiments, many of the embryos in B and C were no longer protected because they re-

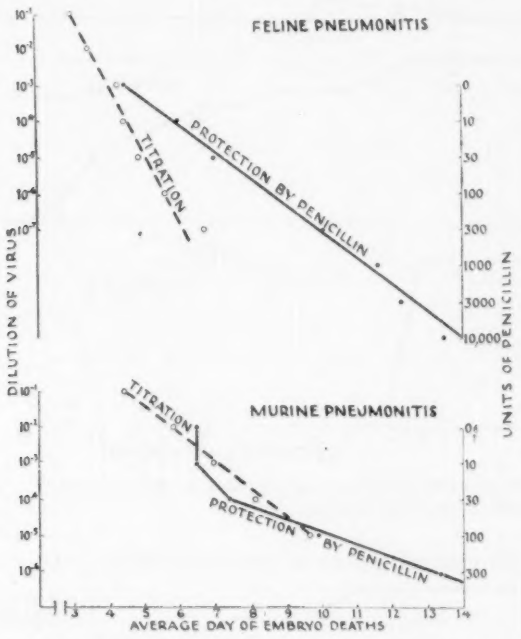


FIG. 1.—Relationship of virulence (indicated by the titration slopes) and susceptibility to penicillin of the agents of feline and murine pneumonitis in chick embryos. See text.

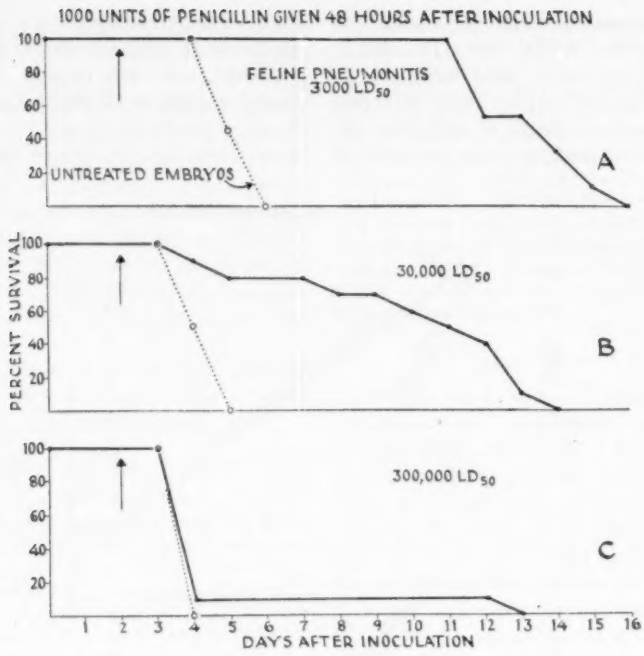


FIG. 2.—Effect of penicillin on varying concentrations of feline pneumonitis virus growing in chick embryos as compared to untreated embryos.

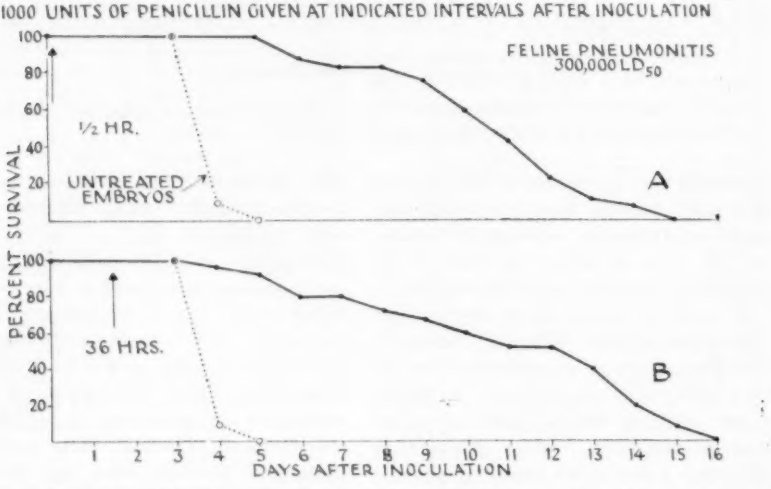


FIG. 3.—Effect of penicillin given at varying intervals of time after inoculation of feline pneumonitis virus into chick embryos.

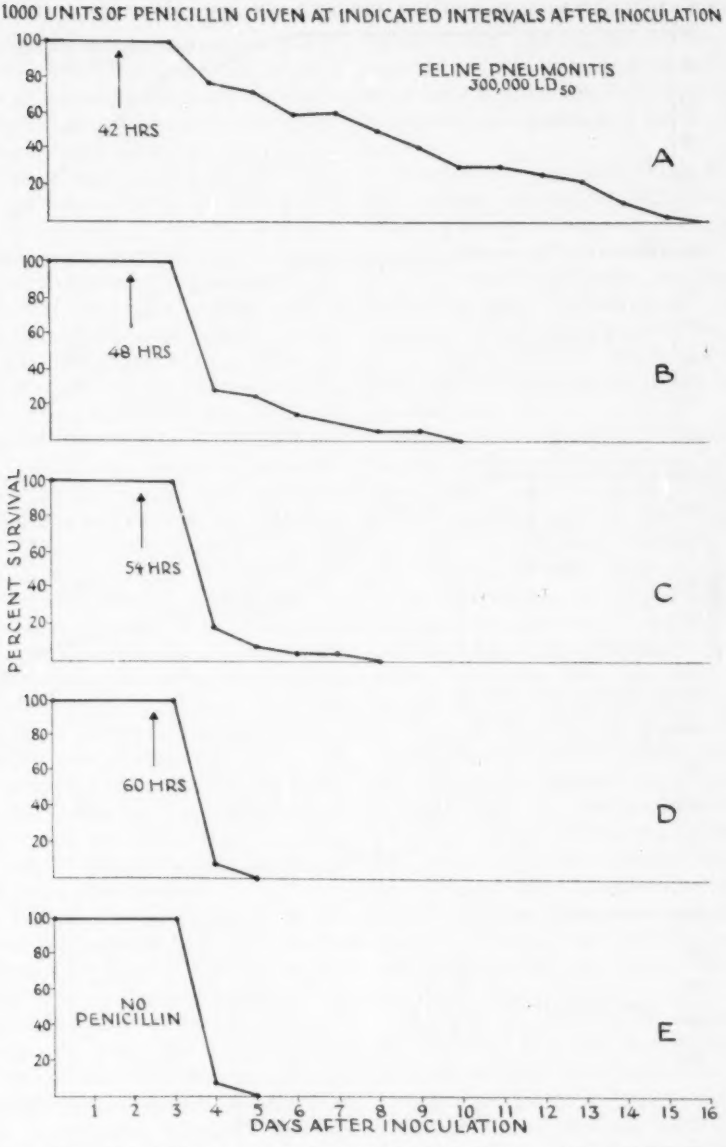


FIG. 4.—Effect of penicillin given at varying intervals of time after inoculation of feline pneumonitis virus into chick embryos.

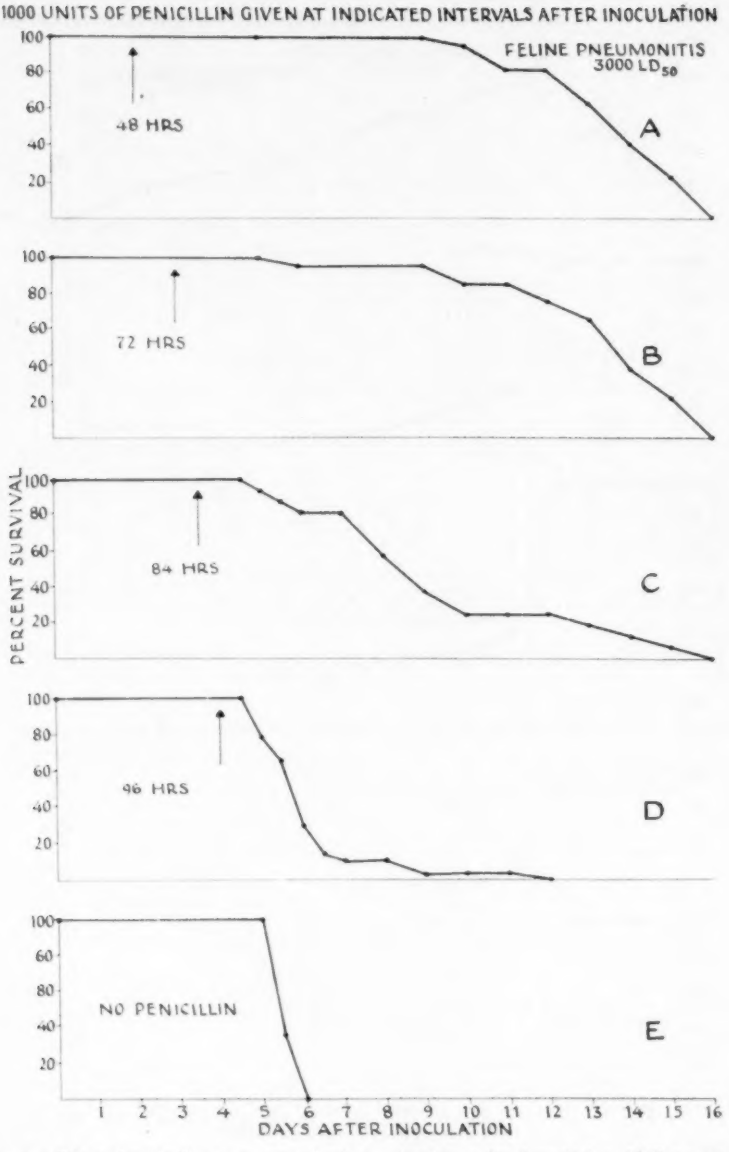


FIG. 5.—Effect of penicillin given at varying intervals of time after inoculation of feline pneumonitis virus into chick embryos.

ceived penicillin too near to their time of death.

3. Effect of delayed administration of penicillin.—In the following experiments an attempt was made to study the effectiveness of penicillin when given at various times during the progress of infection. A large number of chick embryos were inoculated each with 300,000 LD_{50} of virus or an amount sufficient to cause their death in approximately 96 hours, and groups of 30 embryos were given 1000 units of penicillin after ½ hour and every 12 hours from 36 through 60 hours. The degree of protection for 6 of these groups and a control group is shown in figures 3 and 4. Penicillin was just as effective when given at 36 hours as at ½ hour. It was somewhat less effective at 42 hours and was definitely less effective at 48 hours, i.e., 48 hours before the expected death of the embryos. Protection dropped further at 54 hours and disappeared by 60 hours.

Similar results (fig. 5) were obtained with a smaller concentration of virus, i.e., 3000 LD_{50} per embryo, which killed the embryos in about 5½ days. Penicillin given 48 or 72 hours after inoculation protected the embryos equally well but was definitely less protective when given after 84 hours, i.e., approximately 48 hours before the expected death of the embryos. It was practically ineffective when given after 96 hours.

4. The effect of penicillin on the developmental stages of feline and murine pneumonitis.—Since vesicles of murine pneumonitis can be seen in heavily infected embryos several days before the embryos die, they can be studied in treated and untreated embryos. On the other hand, the developmental stages of feline pneumonitis appeared in large numbers in the chick embryos only about 24 to 36 hours before their death, and thus the effect of penicillin on their vesicles could be studied only in embryos which were no longer protected. Because of this, most of the morphological study was carried out on the agent of murine pneumonitis, but the observations were well integrated with those made on the agent of feline pneumonitis.

As previously described (Weiss,1949), the normal developmental cycle of murine pneumonitis lasts approximately 30 to 36 hours, and the synchronism of development is gradually lost during the second cycle. Three days after inoculation of the virus, as shown in figures 6 to 8, all stages are present. At this time penicillin was administered (100 units per embryo).

Under the influence of penicillin, the individual granules making up the clusters and vesicles greatly enlarge without dividing and give rise to forms not seen in untreated embryos. The first stages to be affected are the initial bodies and clusters, which, presumably, are the structures which are growing most actively. Figure 9 shows a cluster which, two hours after the inoculation of penicillin, has granules about twice as large as those seen in figure 6. Four hours after penicillin the smaller vesicles are affected (figs. 10 and 11). It is interesting to note that the granules at the periphery, usually larger than those in the center and more actively dividing, are affected sooner than those in the center. As shown in figure 11 the role of these granules in the development of the vesicle is emphasized by their failure to divide.

Seven hours after the administration of penicillin even the larger vesicles are affected, as shown in figure 12. The granules at the periphery are greatest in size, but some of those in the center are also enlarged. Elementary bodies are still numerous.

The same chain of events continues through 18 hours after treatment. In-

dividual particles develop into plaques several times their original size (fig. 13). The elementary bodies in the center of vesicles gradually decrease in number but do not disappear completely even at later stages.

At about this time, 18 hours after penicillin, some of the plaques are no longer spherical. As shown in figure 14, some are indented and others develop concavities which surround more rounded plaques. Affinity for azur. II stain varies considerably, and many plaques become vacuolated. The irregularity of shape and vacuolization of the plaques progressively increases, as shown by the vesicle in figure 15 three days after treatment. Coincident with these changes, the number of vesicles seen in any one specimen decreases slowly.

The embryos begin to die 5 to 8 days after the administration of penicillin. Prior to their death numerous finely granulated vesicles reappear. The sequence of events from the degenerate vesicles at 3 days (fig. 15) to almost normal vesicles at 8 days (fig. 16) is not entirely clear. Possibly, a few of the larger vacuolated plaques are no longer viable. In fact, as determined, the virus titer declines at a slow rate after administration of penicillin. New vesicles, however, may arise from elementary bodies which, although greatly decreased in number, never completely disappear. Vesicles may also arise from the less degenerate plaques which, possibly, break up and give rise to elementary bodies. The presence of a few plaques in the large finely granulated vesicles seen just prior to the death of the embryos (fig. 16) suggests that some may have broken up to give rise to elementary bodies while others still remained.

As indicated in previous work (Weiss, 1949), the normal individual particles of feline pneumonitis are somewhat smaller than those of murine pneumonitis. They also develop more slowly (in about 48 hours). The vesicle is contained more strictly within the protoplasmic strands of the host cell and often has a long, bizarre appearance. Figures 17 and 18 illustrate a typical normal cluster and vesicle, respectively.

The slower development of murine pneumonitis is paralleled by a slower action of penicillin. Granules of the vesicles and clusters are noticeably enlarged 4 hours after the administration of penicillin. Figures 19 and 20 illustrate the changes which have taken place after 7 hours. Particles in both structures are considerably enlarged but not to the same extent as those of murine pneumonitis. After 18 hours, the increase in size is more pronounced, and irregularly shaped and vacuolated plaques occur (figs. 21 to 23). The infected embryos die shortly afterwards. As indicated above, the 1000 units of penicillin given to each embryo in this experiment did not protect them.

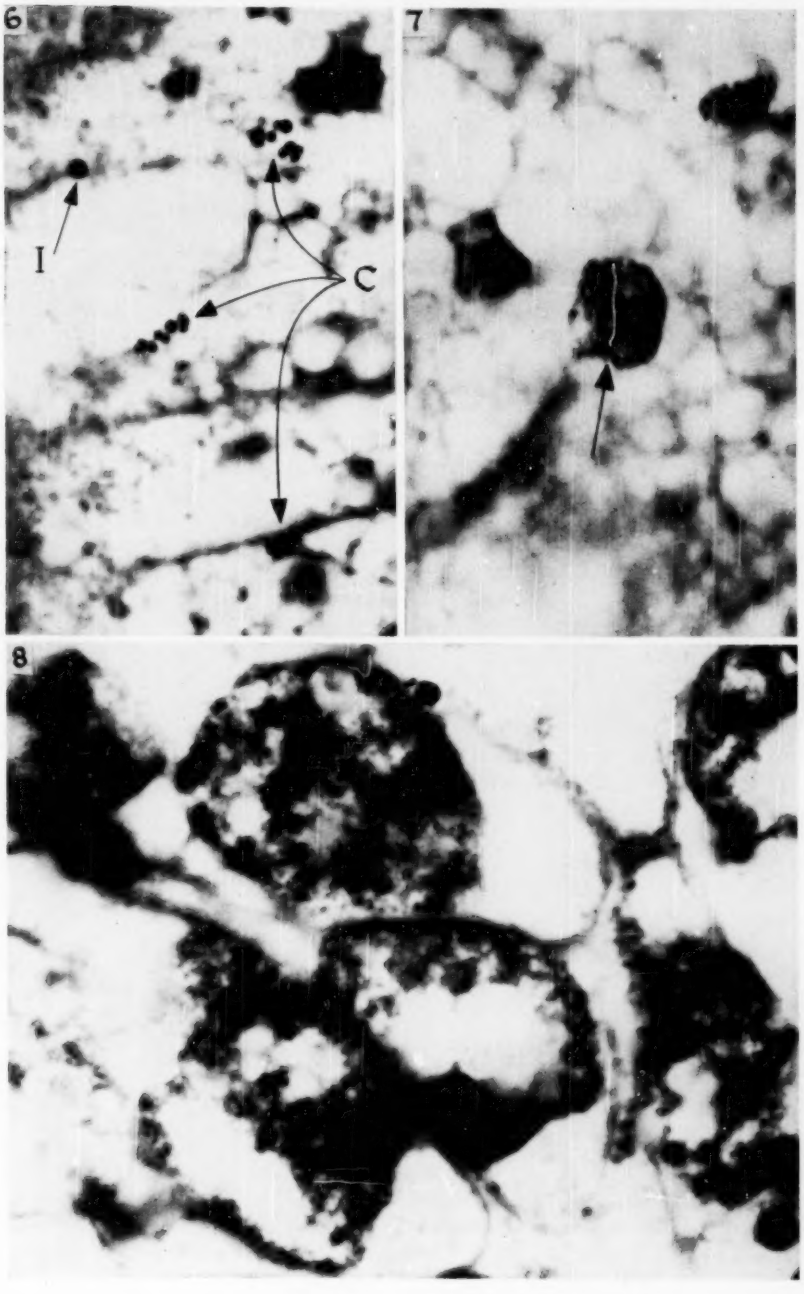
Figures 6 through 23 are photomicrographs of murine and feline pneumonitis viruses growing in the yolk cells of chick embryos. Specimens were taken just prior to administration of penicillin and at various intervals of time thereafter. The tissues were embedded in celloidin, sectioned 6μ thick, and stained with hematoxylin-eosin-azure II. The photomicrographs were taken by Mr. Jean W. Crunelle of the University of Chicago Clinics. Magnification of all photomicrographs is 2000 $\times$.

Figures 6 to 8.—Normal developmental stages of the agent of murine pneumonitis three days after inoculation of the virus.

FIG. 6.—Initial body (I) and clusters(C).

FIG. 7.—Medium-sized vesicle.

FIG. 8.—Large vesicles.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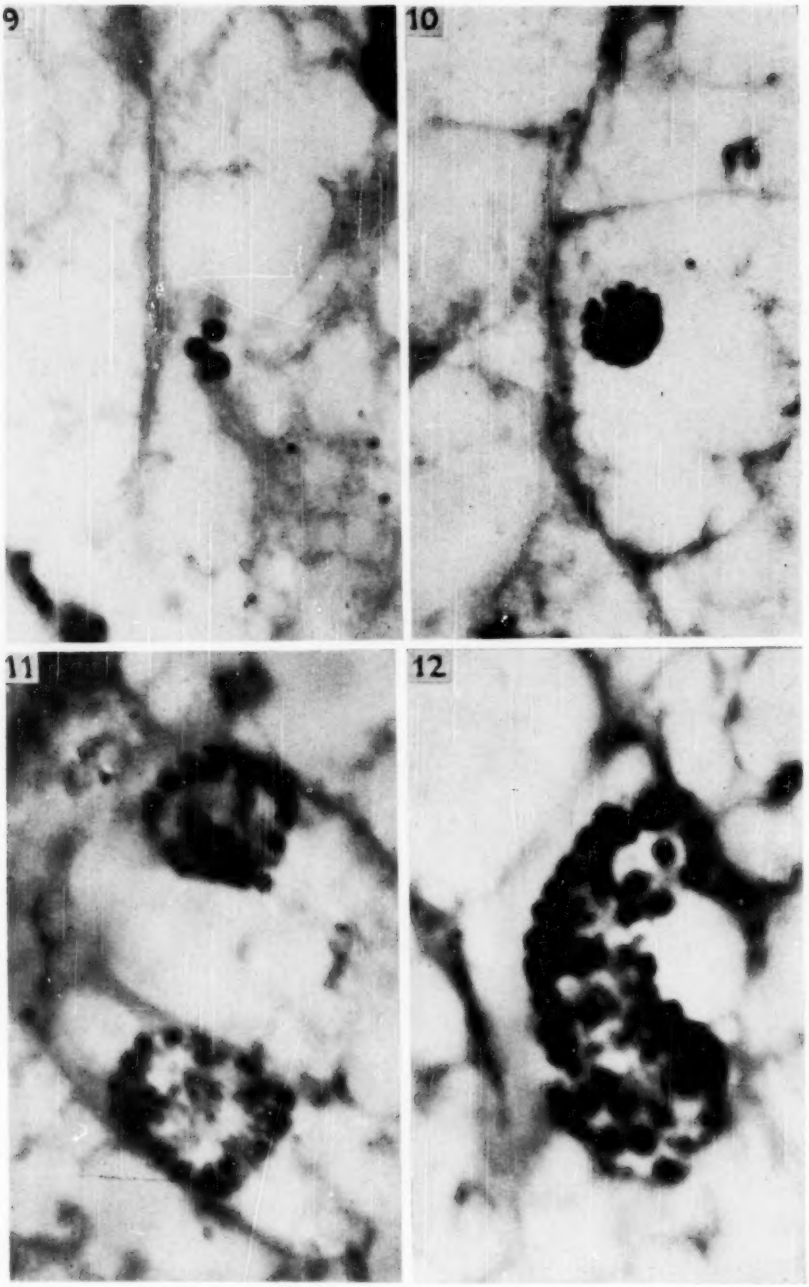


Figures 9 to 16.—Effect of penicillin on the developmental stages of murine pneumonitis virus.
FIG. 9.—Small cluster, two hours after penicillin.
FIG.10.—Small vesicle, four hours after penicillin.
FIG. 11.—Medium-sized vesicles, four hours after penicillin.
FIG. 12.—Large vesicle, seven hours after penicillin

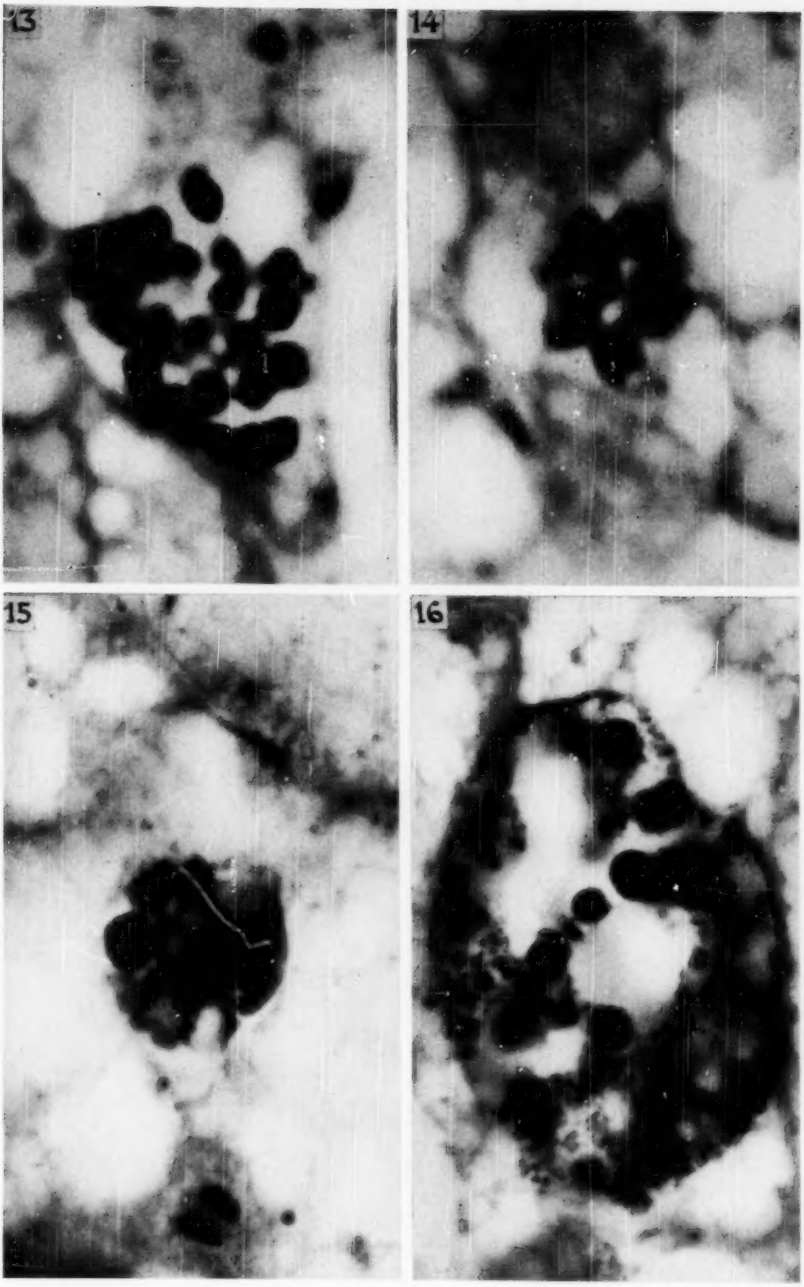
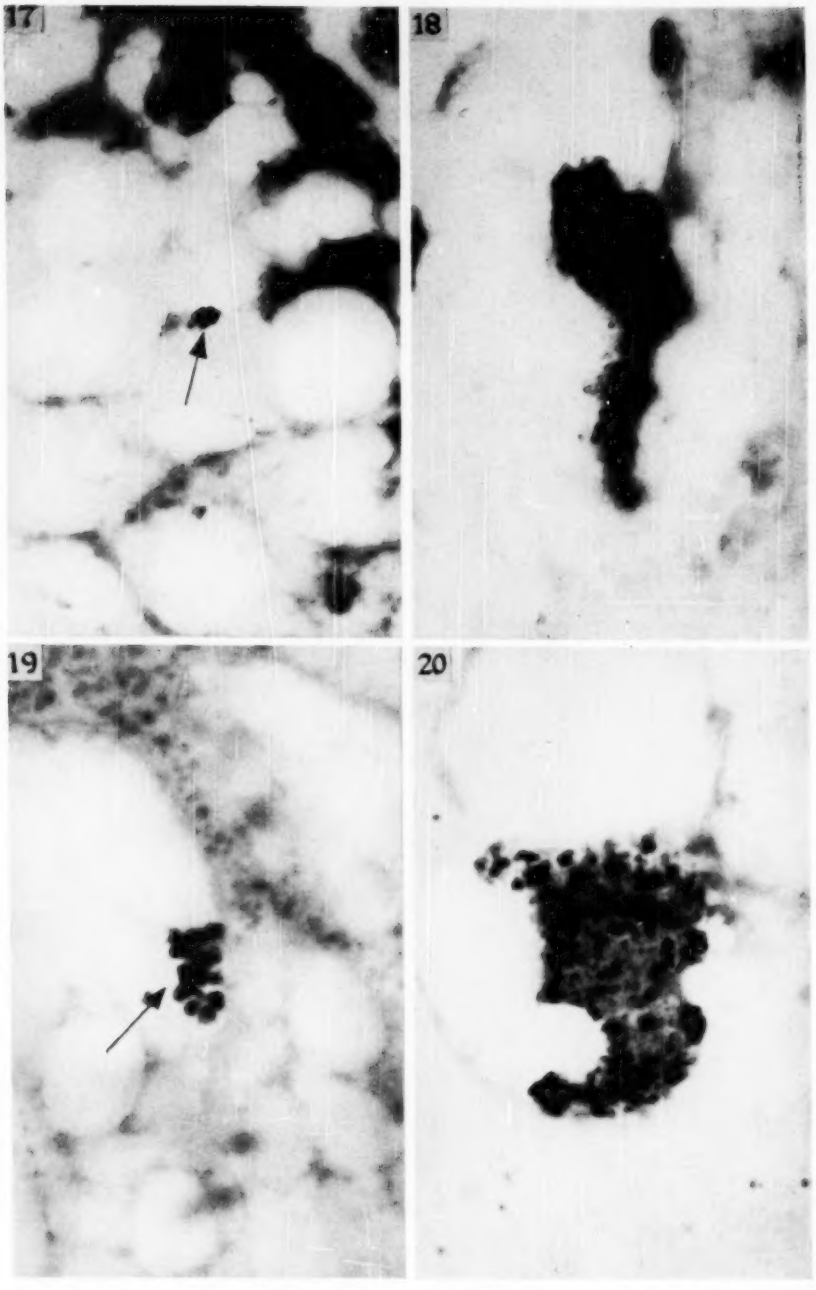


FIG. 13.—Conglomerate of plaques, 18 hours after penicillin.
FIG. 14.—Conglomerate of irregularly shaped plaques, 18 hours after penicillin.
FIG. 15.—Conglomerate of irregularly shaped and vacuolated plaques, three days after penicillin.
FIG. 16.—Large vesicle reappearing after all penicillin has been eliminated and/or destroyed.



Figures 17 and 18.—Developmental stages of the agent of feline pneumonitis prior to the administration of penicillin, four days after inoculation of the virus.

FIG. 17.—Cluster.

FIG. 18.—Vesicle.

Figures 19 to 23.—Effect of penicillin on the developmental stages of feline pneumonitis virus.

FIG. 19.—Cluster, 7 hours after penicillin.

FIG. 20.—Vesicle, 7 hours after penicillin.

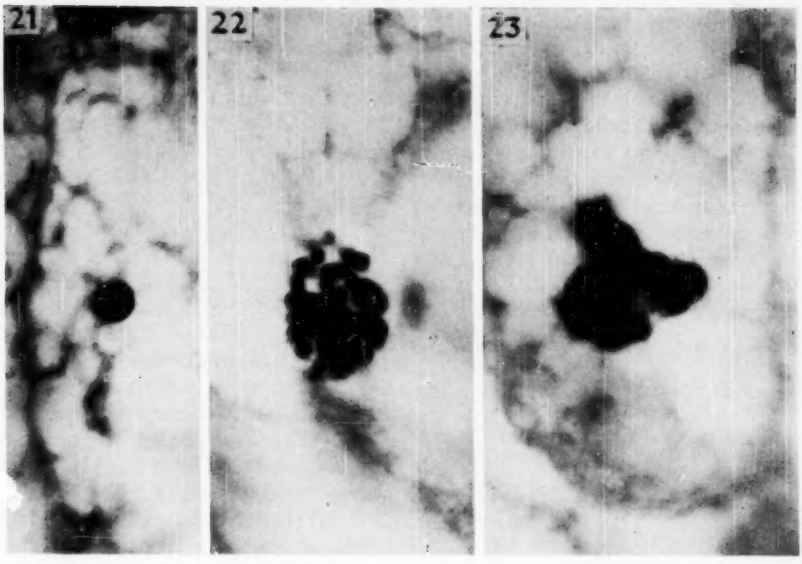


FIG. 21.—Plaque, 18 hours after penicillin.
FIG. 22.—Vesicle, 18 hours after penicillin.
FIG. 23.—Conglomerate of irregularly shaped plaques, 18 hours after penicillin.

DISCUSSION

These results, in regard to the efficacy of penicillin against murine and feline pneumonitis infections, are in general agreement with those obtained by Hamre and Rake (1947) and Eaton et al (1948). It is interesting to note that the delay in ADD of the embryos infected with feline pneumonitis and treated with penicillin is proportional to logarithmic increases of penicillin. This suggests that the delay of ADD is dependent on the exposure of the virus to an effective concentration of penicillin and is dependent on the rate of destruction and/or elimination of the antibiotic from the yolk sac. This rate was not determined in the present study but data are available from the work of Hamre and Rake (1947) and Eaton et al (1948). The latter investigators have found that the rate of destruction of the antibiotic was fairly uniform at various concentrations and that its potency dropped to about $\frac{1}{2}$ in 24 hours. The relationship between rate of destruction of penicillin (solid line) and delay in ADD at the various concentrations (solid circles) is shown in figure 24. The rate of destruction and/or elimination of penicillin appears to be the main factor determining the delay of ADD.

The morphological changes due to penicillin are comparable to those described for bacteria (Pratt and Dufrenoy, 1947). Although the fate of the abnormally developed virus has not been fully determined, it appears that many particles are injured by the process of faulty development. This is indicated by their bizarre appearance, possibly due to lesser consistency, their lower affinity for stains, and vacuolization, and is also confirmed by the decline in virus titer (Gogolak and Weiss, 1950). Eaton et al (1948) have observed

that the greater decrease in virus titer occurs two to three days after administration of penicillin or at the peak of the irregularity of shape and vacuolization as seen in this study.

The experiments illustrated by figures 2 to 5 clearly indicate that within wide limits the effect of penicillin is unrelated to virus concentration. In some experiments various concentrations of virus were inoculated and penicillin administered at one interval of time. In other

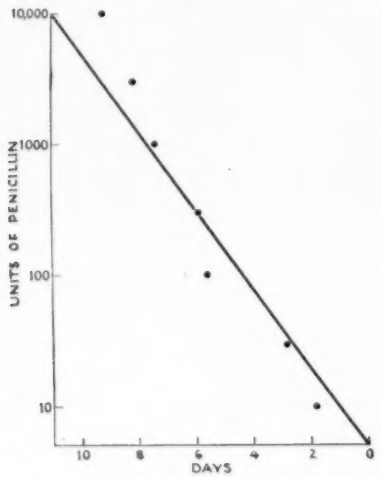


FIG. 24.—Relationship between rate of destruction and/or elimination of penicillin (solid line) and prolongation of ADD of embryos infected with the agent of feline penumonitis (solid circles). See text.

experiments penicillin was given at various intervals of time after inoculation of the same amount of virus. Obviously, the concentrations of virus differed at the various intervals of time that the penicillin was administered.

However, a time is reached in the course of infection of the embryos, 48 hours before their deaths, when penicillin is no longer protective. It does not seem probable that by that time irreversible changes have already occurred in the chick embryos. Aureomycin, for example (Gogolak and Weiss, 1950), is still effective when given well beyond that time. Furthermore, Moulder and Weiss (1951) have shown that the infected cells maintain a normal respiratory rate up to the time of death of the embryos.

The morphological changes occurring in the developmental stages of the virus (fig. 9 to 16, 19 to 23) offer an explanation for the time limit of effectiveness of penicillin. Under the influence of penicillin, the virus, though not dividing, continues to grow, and, presumably, continues to damage the host cells. Forty-eight hours before the death of the embryo, therefore, when penicillin is markedly less efficacious, the virus has invaded enough cells to cause the death of the embryo as soon as each particle has grown to capacity. This conclusion seems justified since the developmental cycle is actually 48 hours.

The results clearly indicate that in evaluating the efficacy of chemotherapeutic drugs, the mechanism of development of the viruses in the parasitized cells should be considered.

SUMMARY

1. The effect of penicillin on the agents of feline and murine pneumonitis grown in the yolk sacs of chick embryos was studied. Although the two agents appeared equally susceptible, the effect on the more virulent feline pneumonitis virus could be more accurately determined. The agent of murine pneumonitis, however, because of the early appearance of its vesicles in tissue sections, proved very valuable for morphological investigations.

2. Penicillin, in concentrations of 10 to 10,000 units, delayed the average day of death (ADD) of the infected embryos. The delay was proportional to logarithmic increases of penicillin,

suggesting that it was determined by the rate of destruction and/or elimination of the antibiotic. Protected embryos eventually died and were shown to have heavy viral infections.

3. The efficacy of penicillin, although relatively independent of viral concentration and time of administration, stopped abruptly when administered less than 48 hours before the expected death of the embryos, or the length of a complete developmental cycle.

4. A microscopic study revealed that penicillin prevented division of the virus particle but allowed an abnormal type of growth to take place. The granules making up the initial bodies, clusters, and vesicles developed into large irregularly shaped, vacuolated plaques.

5. It is suggested that the virus developing abnormally under the influence of penicillin continues to injure its host cell. Therefore, penicillin is effective only when given before a large number of host cells are invaded.

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THE EFFECT OF ANTIBIOTICS ON AGENTS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP

II. THE EFFECT OF AUREOMYCIN

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Aureomycin (Duggar, 1948) has been found to be very effective against agents of the psittacosis-lymphogranuloma group in experimental infections (Wong and Cox, 1948; Wagner, 1949; Wells and Finland, 1949) as well as in numerous clinical trials as reported, among others, by Woodward (1949), Brainerd et al (1949) Wright et al (1948), Runyan et al (1949), and Robinson (1950). It is now generally agreed that aureomycin will replace penicillin and the sulfonamides in the treatment of psittacosis and lymphogranuloma venereum.

The present study was prompted by the belief that the greater effectiveness of aureomycin over penicillin would be reflected in its influence on the biology of the viruses. Therefore, this investigation closely paralleled the preceding one (Weiss, 1950b). The agent of feline pneumonitis was used throughout.

MATERIALS AND METHODS

The egg-adapted strain of feline pneumonitis virus and the procedures of preparing virus pools and inoculating chick embryos were identical to those previously described (Weiss, 1950a and b).

Aureomycin hydrochloride was obtained from the Lederle Laboratories in 100 mg sterile ampoules, as distributed for human intravenous injection. Just prior to its use, the drug was dissolved in sterile distilled water to make a stock solution of 10 mg of aureomycin per ml. Subsequent dilutions were prepared to contain the desired amount in 0.25 ml of fluid. Aureomycin in quantities up to 2.5 mg per embryo, as used in these experiments, did not prevent normal development and hatching of chick embryos.

For histological investigations, enough virus (1000 LD_{50}) was given to kill unprotected embryos in 5 to 6 days. Aureomycin (0.15 mg per embryo) was administered 4 days afterwards, and 2 specimens of yolk sacs were taken at each of the following time intervals: ¼, ½, 1, 3, 6, 9, 12, 24, 36 hours, and 2, 3, 3½, 4, 5 and 5½ days. In addition, untreated control specimens were taken at daily intervals after 3 days. The histological techniques are described in the preceding work (Weiss, 1950b).

RESULTS

1. Effect of varying concentrations of aureomycin.—A large number of chick embryos were each inoculated with 1000 LD_{50} of feline pneumonitis virus. One hour later groups of 10 embryos were given various concentrations of aureomycin. As shown in figure 1, the antibiotic in concentrations of at least 0.025 mg per embryo delayed the average day of death (ADD) of the embryos. The delay of ADD was in direct proportion to the amount of aureomycin given. For example, 0.1 mg and 0.2 mg delayed the ADD of infected embryos 3.5 and 6.6 days, respectively. Protected embryos, however, eventually succumbed to an infection as heavy as in controls, as determined by the final titer of virus in dying embryos. Large amounts of aureomycin,

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such as 2.5 mg per embryo, protected the embryos beyond hatching time (fig. 2B).

2. Effect of aureomycin on varying concentrations of virus.—The relationship of the concentration of virus to efficacy of aureomycin was studied by varying the amount of virus (10, 1000, and 100,000 LD_{50}) as well as the amount of aureomycin. As in the previous experiments the antibiotic was injected 1 hour after the inoculation of the virus. The results are shown in figure 2. Each column represents the average of 10 embryos.

It is apparent that the efficacy of aureomycin is remarkably independent of virus concentration. The untreated embryos and those receiving the smallest amount of the antibiotic (0.0025 mg) died at different intervals of time, 4.1, 5.3, and 7.4 days, depending on the concentration of virus they received. However, 0.025 mg delayed death for about 1 day in all three cases. Larger amounts of aureomycin (0.25 mg) caused delays of 6.1 days in C and 7.4 days in B. In A the prolongation was at least 7 days, and, because of the low concentration of virus, this delay was long enough to allow the embryos to hatch. Large amounts of aureomycin, 2.5 mg, allowed all embryos to hatch, even those receiving the heavy concentration of virus.

3. Effect of delayed administration of aureomycin.—This experiment involved 5 groups of 25 embryos which received 100,000 LD_{50} or enough virus to kill the embryos in 4 to 4½ days. Four groups were treated with 0.25 mg of aureomycin per embryo 2, 72, 84, and 96 hours thereafter. Results are given in figure 3. Twenty-five embryos were used for testing each interval. The effectiveness of aureomycin did not change when administration was delayed from 2 to 72 hours. However, a marked drop in effectiveness occurred when the antibiotic was given 84 hours after inoculation of the virus, but some embryos were still definitely protected at this time. Since untreated embryos began to die shortly after this interval of time and were all dead 24 hours later, it appears that aureomycin is still effective when given approximately 24 hours before the expected death of the embryos.

A second group of embryos receiving 1000 LD_{50} of virus was also inoculated with aureomycin at various intervals of

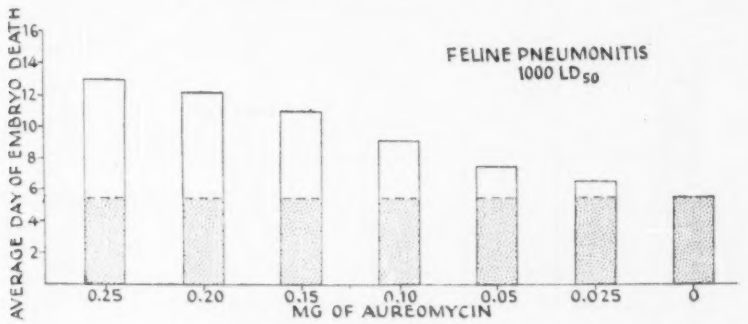

FIG. 1.—Average day of death of groups of embryos inoculated with the same concentration of virus and varying amounts of aureomycin. Stippled area indicates time of death of the control embryos, clear area length of protection.

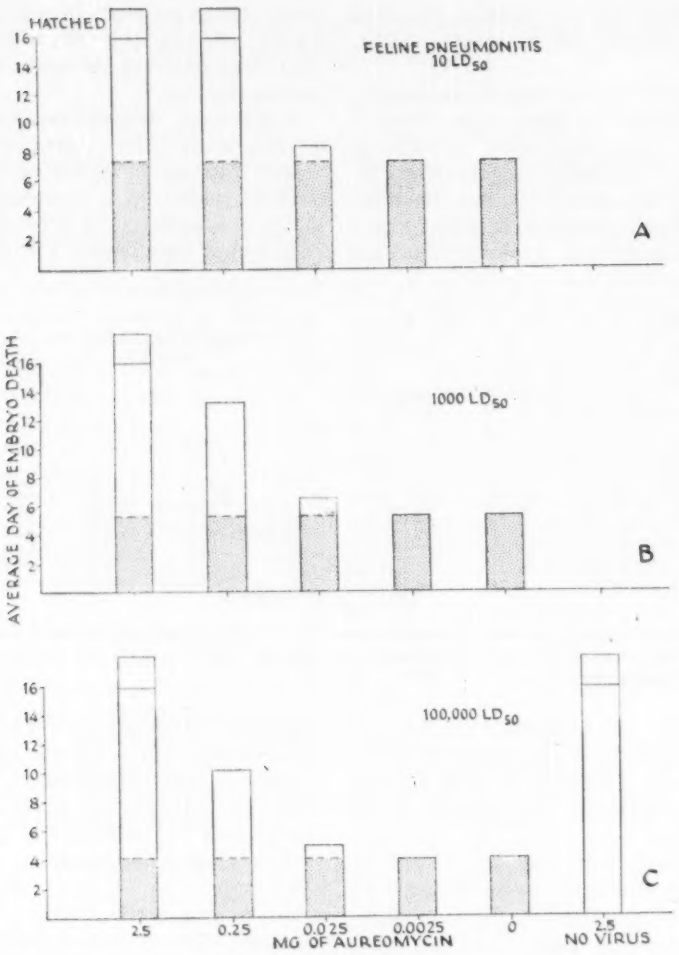

FIG. 2.—Average day of death of groups of embryos inoculated with varying concentrations of virus and of aureomycin. Stippled and clear areas as in figure 1.

time. The results are shown in figure 4. The untreated embryos died in 5 to 6 days. Administration of aureomycin delayed up to 96 hours after inoculation of virus protected the embryos as well as that given at previous intervals. A moderate drop in efficacy was noted when aureomycin was given 108 hours after the virus. This experiment indicated again that aureomycin was effective up to 24 hours before the death of the embryos.

4. The effect of aureomycin on the developmental stages of feline pneumonitis.—Under the influence of aureomycin the individual granules making up the clusters and vesicles of the virus enlarged, as they did under the influence

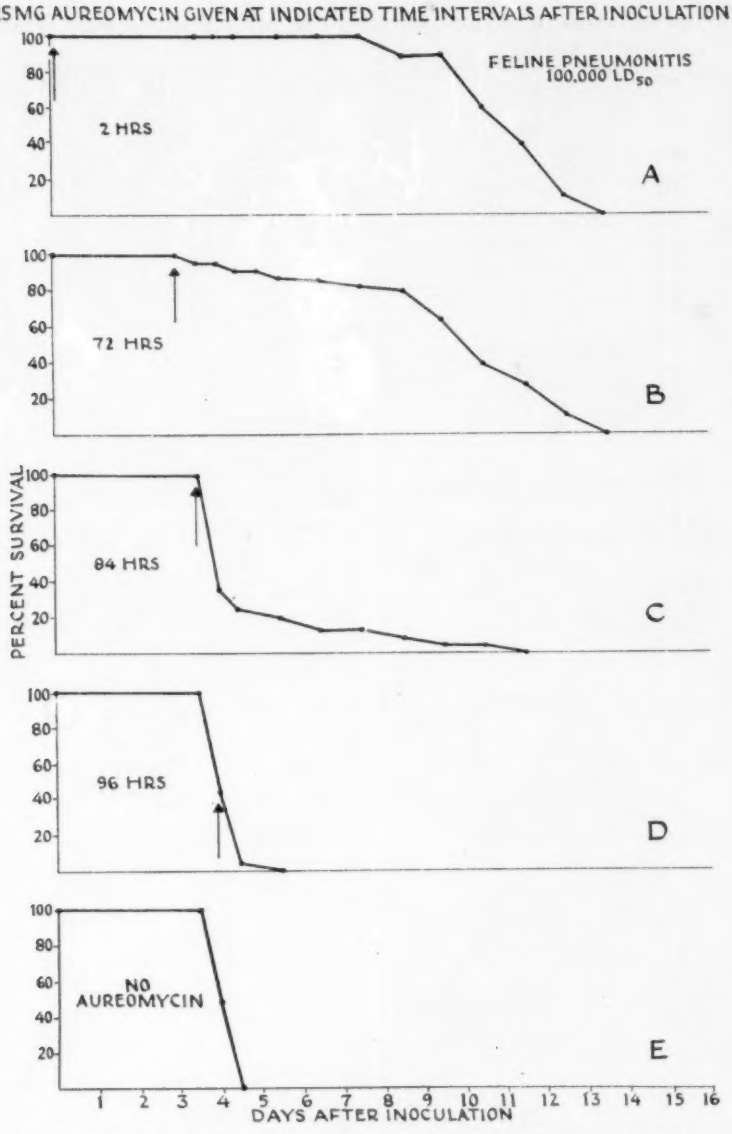

FIG. 3.—Survival of infected embryos given the same amount of aureomycin at varying intervals of time. Note sharp drop in efficacy when aureomycin is administered after 84 hours.

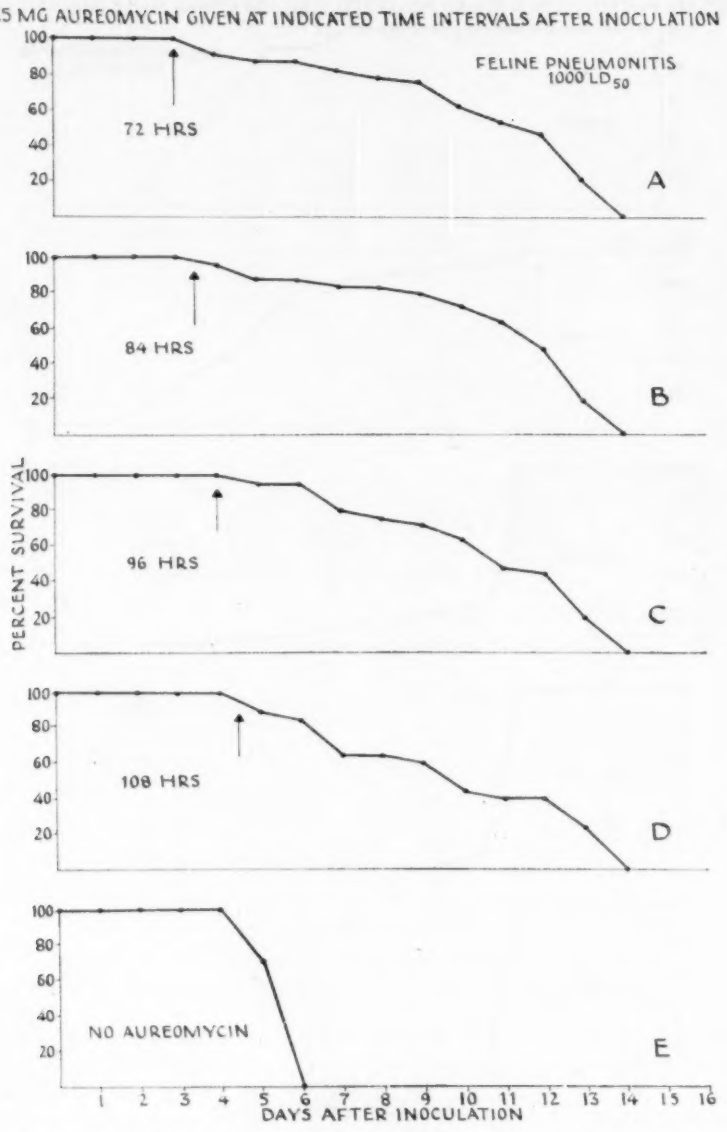

FIG. 4.—Survival of infected embryos given the same amount of aureomycin at varying intervals of time. Note drop in efficacy when aureomycin is administered after 108 hours.

Figures 5 through 12 are photomicrographs of feline pneumonitis vesicles growing in the yolk sacs of chick embryos and exposed to aureomycin for various lengths of time. The tissues were fixed in Zenker-formol, embedded in celloidin, sectioned 6μ thick and stained with hematoxylin-eosin-azure II. The photomicrographs were taken by Mr. Jean W. Crunelle of the University of Chicago Clinics. Magnification 2000 ×.

FIG. 5.—Three hours after aureomycin.

FIG. 6.—Twelve hours after aureomycin.

FIG. 7.—Thirty-six hours after aureomycin.

FIG. 8.—Three days after aureomycin.

of penicillin (Weiss, 1950b), but at a much slower rate. The vesicle in figure 5 is representative of those exposed to aureomycin for 3 hours and cannot be distinguished from control vesicles. Twelve hours after inoculation (fig. 6) an increase in size was barely noticeable. Individual particles grew 2 to 3 times in diameter by 36 hours, and the vesicles at that stage resembled those exposed to penicillin for 7 hours (Weiss, 1950b). By 3 days the granules developed into small plaques and resembled structures exposed to penicillin for 12 to 18 hours.

In this experiment enough aureomycin was given to delay the death of the embryos for 4 to 6 days. Figures 9 to 12 illustrate the morphology of vesicles during the period when most of the aureomycin had disappeared and the virus had begun to develop again, causing the embryos to eventually die. Figure 9 illustrates the morphology of a vesicle in a living embryo after treatment with aureomycin. The vesicle is mostly made up of plaques. The vesicle in figure 10 is from a dead embryo of the same time interval. Many particles about the size of elementary bodies are already present. The plaques, on close inspection, reveal a granular structure, indicating that they are about to break up and give rise to elementary bodies. A similar phenomenon is well illustrated in figures 11 and 12, taken, respectively, from a living and dead embryo $5\frac{1}{2}$ days after the administration of aureomycin. The vesicle in figure 11 still contains many plaques, but some of them are in the process of breaking up. The vesicle in figure 12 appears identical to those in the untreated embryos.

In general, only a moderate number of yolk cells were infected prior to the administration of aureomycin. For a period of about 4 days after the inoculation of aureomycin, the number of vesicles remained approximately constant. Then, as aureomycin was eliminated or destroyed, the infection became heavier and resembled that of dying, untreated embryos.

DISCUSSION

1. The results of these experiments appear to indicate that aureomycin is not virocidal but only inhibits the development of the virus. However, in view of the instability of the antibiotic at 37 C, at neutral or slightly alkaline pH, or in the presence of protein (Price et al, 1948; Paine et al, 1948), it is difficult to understand why a relatively small amount of antibiotic, such as 0.25 mg per egg, should exercise an inhibitory action lasting over 7 days. It is interesting to note that Klein et al (1950) similarly protected mice infected with type I pneumococci for prolonged periods by giving aureomycin intramuscularly, instead of intravenously. It was noted in the course of the experiments that the drug becomes highly insoluble at a neutral or slightly alkaline pH and in a medium such as egg yolk. It appears that in the chick embryos, except for the very low drug concentrations, a precipitate is formed and the drug exists in a soluble and an insoluble phase. Possibly only the soluble phase is subject to deterioration or excretion and is being continuously replenished from the insoluble phase. So long as precipitate is present the concentration of soluble aureomycin remains constant, and the deterioration of the total amount of aureomycin proceeds at an arithmetic rate. As soon as all the precipitate is exhausted, the deterioration continues at a logarithmic rate, as shown in figure 13.

The data used to determine the rate of destruction of aureomycin in figure 13 were derived from the prolongation of chick embryo life observed in these ex-

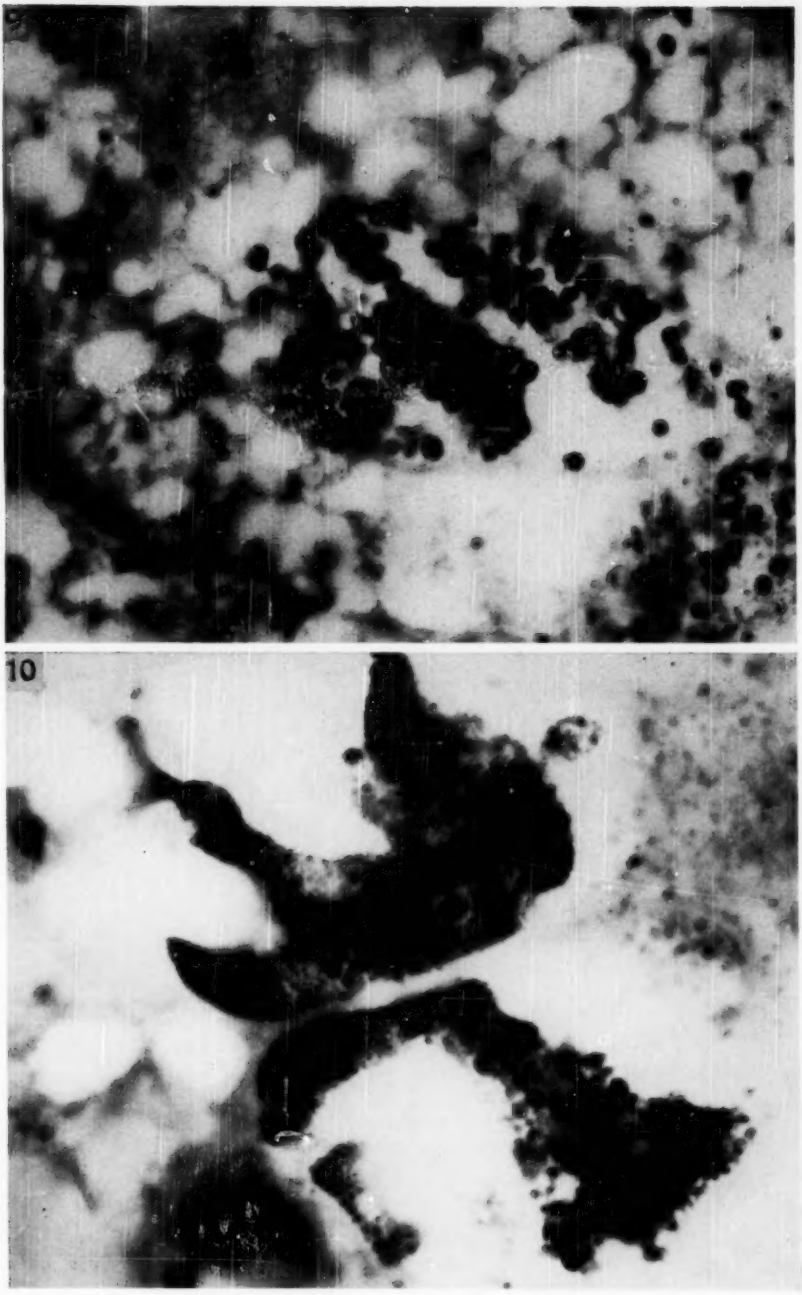

FIG. 9.—Four days after aureomycin, from a living embryo.
FIG. 10.—Four days after aureomycin, from an embryo which had died a few hours previously.

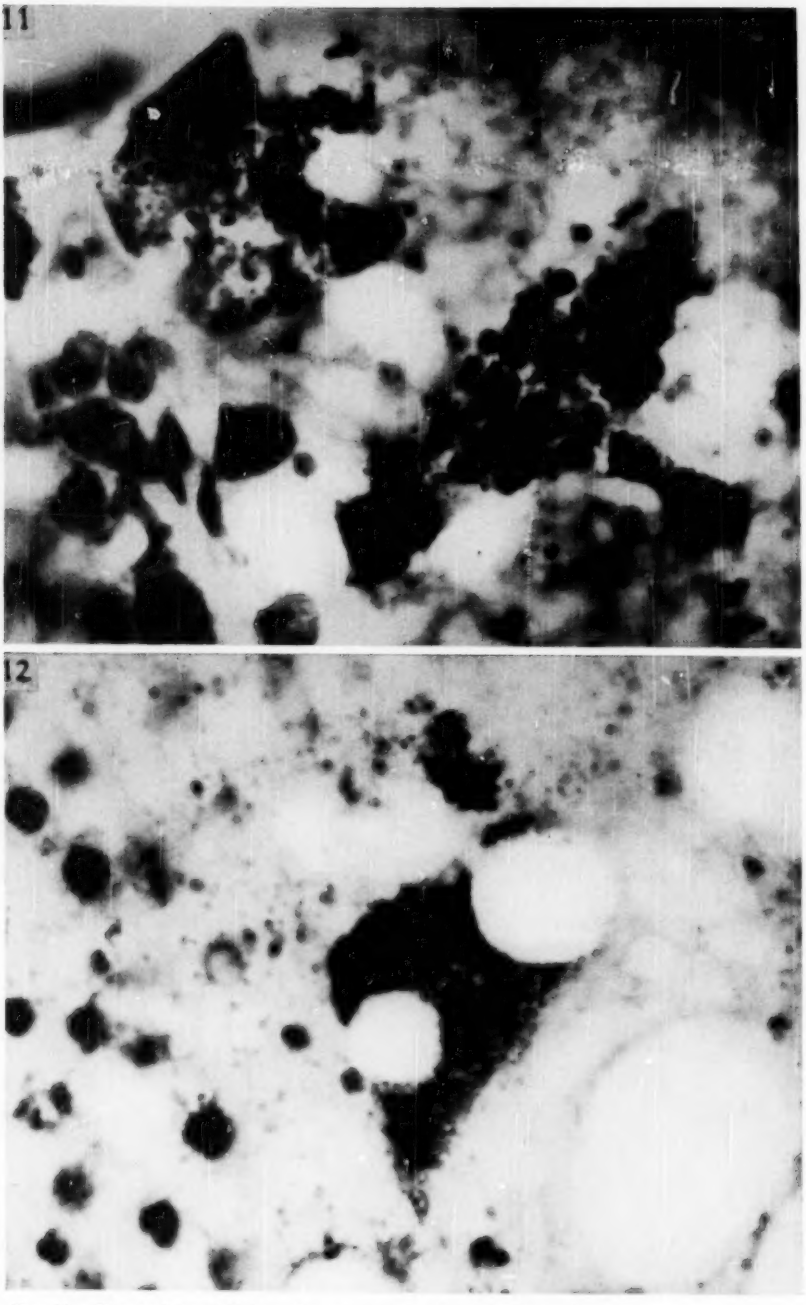

FIG. 11.—Five and one-half days after aureomycin, from a living embryo.

FIG. 12.—Five and one-half days after aureomycin, from an embryo which had died a few hours previously.

periments (solid circles) and from the rate of destruction of aureomycin (half life of 12 hours) at 37 C in plasma

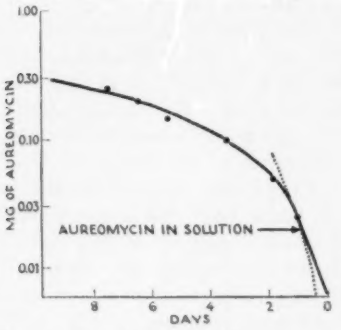

FIG. 13.—Relationship between rate of destruction and/or elimination of aureomycin (solid line) and prolongation of ADD of embryos infected with the agent of feline pneumonitis and given a single dose of aureomycin one hour later (solid circles). See text.

of aureomycin (solid line, fig. 13) can explain how a drug generally more unstable than penicillin and considerably less potent (50 mg of aureomycin are about as effective as 6 mg of penicillin, Weiss, 1950b) can, in most cases, become more efficacious.

2. The efficacy of aureomycin is remarkably independent of virus concentration as shown by varying the concentration of virus and the time of administering the antibiotic. These results agree with those of Bliss and Todd (1949), who noticed that aureomycin is among the antibiotics least affected by the size of the bacterial inoculum.

3. In sharp contrast with penicillin (Weiss, 1950b), the administration of aureomycin can be delayed until 24 hours before the death of the embryos, or one-half the time of a developmental

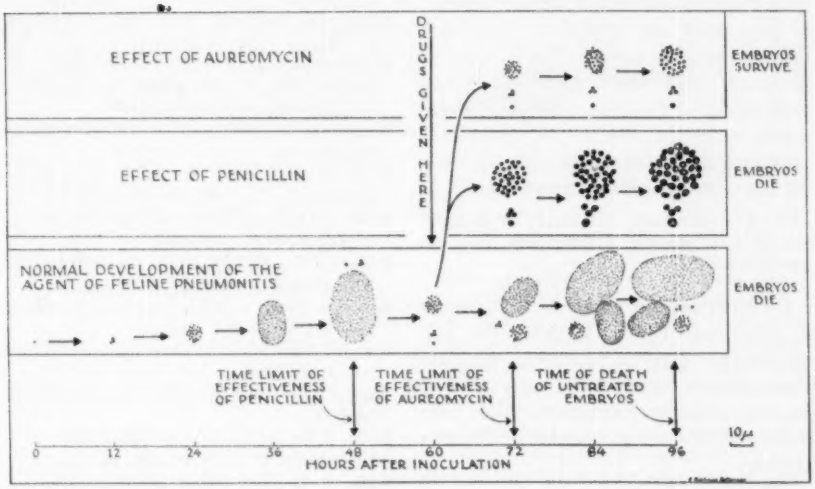

FIG. 14.—Comparative effects of penicillin and aureomycin on the developmental stages of the agent of feline pneumonitis.

gathered by Paine et al (1948). It follows that, as long as a precipitate is present, 20 mg of aureomycin are available in the soluble state to the embryo. This hypothesized rate of destruction

cycle. This indicates that aureomycin is a more powerful inhibitor of viral activity than penicillin. In fact, penicillin prevents division of the initial body, but growth continues at the same rate

and large plaques are formed (Weiss, 1950b). Aureomycin, on the other hand, prevents initial body division and almost completely suppresses growth, which is allowed to proceed only at a very slow rate. The plaques produced under the influence of penicillin appear to be just as injurious to the host as the normal vesicles, while the slow growing vesicles exposed to aureomycin are not. The comparative efficacy of the two antibiotics and their mechanism of action are schematically summarized in figure 14.

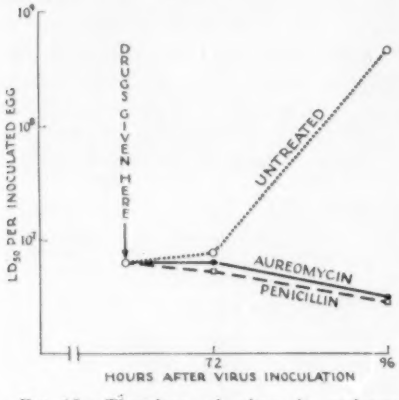

FIG. 15.—Titrations of virus in embryos treated with penicillin, aureomycin, and untreated controls.

Direct evidence that virus division is prevented by both penicillin and aureomycin is given by the experiments illustrated in figure 15. Under the influence of either antibiotic the titer of the virus remains constant or declines slightly.

SUMMARY

1. Aureomycin in concentrations of 0.025 to 0.25 mg significantly prolongs the life of chick embryos infected with the agent of feline pneumonitis. The delay of average day of death (ADD) is directly proportional to the amount of aureomycin given, indicating that in the chick embryo the antibiotic is destroyed at a constant rate.

2. The effect of aureomycin is markedly independent of virus concentration (from 10 to 100,000 LD_{50}) and time of administration. The antibiotic is effective up to 24 hours before the expected death of the embryos.

3. Under the influence of the antibiotic initial body division is prevented and growth is greatly retarded. The granules making up the clusters and vesicles slowly enlarge and eventually become small plaques.

4. It appears that aureomycin is a more powerful inhibitor of the activity of feline pneumonitis virus than penicillin.

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THE EFFECT OF SERUM ON THE ACTIVITY OF THE POLYMORPHONUCLEAR LEUKOCYTES OF THE GUINEA PIG

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The close association of the circulating polymorphonuclear leukocytes with the defense mechanism of the body is well understood. It is also recognized that the effective extravascular participation of the blood leukocytes in the defense of the host depends upon the consummation of four activities of which these cells are capable, namely (1) diapedesis, (2) ameboid migration within the tissues, (3) phagocytosis of invading organisms, and (4) digestion of engulfed organisms.

Since the general description of the phagocytic process by Metchnikoff, many studies have been made of the activities of the circulating leukocytes. It was noted quite early that serum contained substances which enhanced the engulfment of bacteria and other particulate matter by the white blood cells, and much evidence has been accumulated concerning the nature and action of normal and immune serum opsonins. Less interest has been manifested in the effect of serum on the other activities of leukocytes, especially its effect on their motility. More particularly, little attention has been given to the indirect effect of serum or serum components on phagocytosis through an alteration of motility.

Friedemann and Schönfeld,[1] using rabbit leukocytes, noted a marked diminution or complete cessation of motion when serum was omitted from the system under study. These investigators also reviewed a number of earlier related observations. More recently Delaunay and Pages[2] reported failure to observe either chemotaxis or motility of guinea pig white blood cells in the absence of serum.

The present investigation was undertaken to study the relationships of serum to ameboid motion of leukocytes. It also seemed desirable to determine to what extent the phagocytic process was correlated with changes in pseudopod formation and motility. To accomplish these purposes, polymorphonuclear leukocytes were observed in living, unstained preparations with the aid of darkfield illumination, temperature being maintained at 37 C. Studies were made both with and without the addition of phagocytizable particles. In this manner opsonic effects could be ruled out.

METHODS

Leukocyte suspensions were obtained according to the technique used by Nungester and Ames.[3] Such a procedure, which involves the production of an exudate in the peritoneal cavity of guinea pigs in response to the injection of Locke solution, yields a cell suspension containing 80 to 85% polymorphonuclear leukocytes.

In the studies involving only ameboid motion and related phenomena, the specimens for microscopic examination were prepared by adding 0.02 ml of washed, packed leukocytes to 0.5 ml of the serum sample or solution used to replace serum.

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After thorough mixing, a drop of this material was placed within a ring of petrolatum on a glass slide and a coverslip superimposed. These slides were then incubated at 37 C for 15 minutes prior to microscopic examination.

In later experiments, in which studies of phagocytosis were made concurrently with observations of ameboid motion, the procedure was modified as follows: To 0.1 ml of a suspension of leukocytes in Locke solution (45,000 to 50,000 cells per cmm) was added 0.8 ml of the serum or solution under investigation. The tubes were thoroughly agitated and allowed to stand for 15 minutes at 25 C to permit the test substance to act on the cells before bacteria were added. At the end of this period 0.1 ml of a standard suspension of *Bacillus anthracis* (turbidity was adjusted to 30% transmission of light in a photoelectric nephlometer using a green filter), heat-killed, was added and mixed throughly with the leukocytes. A drop of this mixture was placed on a slide and the procedure described above was then followed.

Microscopic observation of the living white blood cells was facilitated by the use of darkfield illumination. The microscope was kept at 37 C ±1. The diameter of the cells was measured with a calibrated ocular micrometer. Direct observation yielded data on amoeboid motion, pseudopod formation, Brownian motion of the cytoplasmic granules, adhesion of the cells to the slide and coverslip, the tendency of the leukocytes to clump, and morphological changes in the cells. Such observations were evaluated as 0, 1, 2, 3, or 4. In each instance 4 was the maximum value and was based on a series of examinations of leukocytes in normal guinea pig serum under optimal conditions. One, 2, and 3 were intermediate values. The data presented are, therefore, subject to the usual errors inherent in such a procedure. Modifications of the above technique made possible the quantitative determination of phagocytosis.

EXPERIMENTAL

Factors in normal guinea pig serum responsible for leukocyte activity

Inorganic ions of the serum.—Guinea pig leukocytes, washed free of serum, were suspended in Locke solution to determine whether or not a balanced solution of inorganic ions as supplied by Locke solution would be capable of supporting the activity of the leukocytes at the same level as normal serum. Another portion of the same specimen of cells was suspended in normal guinea pig serum to serve as a control. The results of these experiments are summarized in table 1; the figures are the mean values for a series of tests. It will

TABLE 1.—*Activity of guinea pig leukocytes in Locke solution.*

Leukocytes suspended in:	Locke solution	Normal guinea pig serum	Difference of means
			Standard error of difference
Opacity of cytoplasm	3.0*	0.2	9.4
Brownian motion of granules	1.3	3.7	9.0
Pseudopod formation	0.4	2.4	5.8
Motility	0.1	2.1	5.9
Diameter (in microns)	10.1	14.6	8.3
Degenerative changes	1.4	0.3	4.1
Adhesion to slide	1.5	3.9	7.3

* The figures represent mean values of 19 measurements. Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

be noted that in no respect was the activity in Locke solution comparable to that in serum. Cells in the former medium underwent marked degenerative changes, were almost immobile, Brownian motion of the granules all but ceased, and adhesion to the glass surfaces was reduced significantly.

TABLE 2.—*Effect of addition of gum arabic to Locke solution to give same viscosity as serum.*

Luekocytes suspended in:	Locke solution plus gum arabic	Locke solution	Difference of means
			Standard error of difference
Brownian motion of granules	1.0*	1.3	0.4
Pseudopod formation	.0.3	0.4	0.1
Motility	0.0	0.1	1.4
Diameter (in microns)	10.3	10.1	0.4
Degenerative changes	1.7	1.4	0.6

* The figures represent mean values of three measurements. Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

Viscosity of the serum.—A possible objection to the use of a solution such as that of Locke in place of serum is that the solution is markedly less viscous than serum. Indeed, Friedemann and

Schönfeld[1] believed the viscosity of serum was the reason for its ability to support motility. According to their findings leukocytes were as motile in Locke, Ringer, or other balanced salt solution as they were in serum, when 7 or 8% gum arabic was added to the solutions. Tests carried out in the present investigation failed to confirm this conclusion. Moreover, when the viscosity of Locke solution was adjusted to that of serum by the addition of gum arabic (relative viscosities were measured with an Ostwald viscometer), there was no increase in activity of the white blood cells over that in Locke solution alone. The results are presented in table 2.

Complement.—Still another explanation for the activity of leukocytes in normal serum was put forth by Delaunay and Pages.[2] These workers, studying chemotactic attraction of guinea pig white blood cells to starch granules, presented evidence to support the theory that complement was the critical substance.

TABLE 3.—*Effect of heat on guinea pig serum.*

Leukocytes suspended in:	Guinea pig serum		Difference of means
	Heated at 56 C	Unheated	Standard error of difference
Opacity of cytoplasm	0.4*	0.4	0.4
Brownian motion of granules	3.6	3.6	0.4
Pseudopod formation	2.2	2.1	0.1
Motility	1.5	1.6	0.7
Diameter (in microns)	14.2	14.5	1.0
Degenerative changes	0.2	0.1	1.5
Adhesion to slide	3.5	3.6	1.0
Percentage phagocytosis	45.6	61.7	1.5

* The figures represent mean values of 56 measurements. Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

In an attempt to verify this conclusion a sample of fresh, normal guinea pig serum was divided into two equal portions; one portion was heated at 56 C for 30 minutes to inactivate complement, and the other was a control. The data presented in table 3 clearly show that any effect of complement on motility is extremely doubtful. In the few tests run with anthrax bacilli present, no effect on phagocytosis was noted. No attempt was made to study the relationship of complement to chemotaxis.

Effect of serum from other species: specific antileukocyte serum and normal serum

Deleterious effect of fresh serums.—A specific antiserum against guinea pig leukocytes, prepared by injecting rabbits intraperitoneally with a washed suspension of cells from peritoneal exudate of guinea pigs, was available. When this antiserum was tested on homologous leukocytes by means of the warm stage technique described above, it was found that the cells were nonmotile, abnormal in appearance, and showed clumping in high dilutions of the antiserum. When control tubes containing normal rabbit serum in place of the antiserum were examined, however, the results were identical except that clumping was not as pronounced. Repeated tests with normal rabbit serum, using normal guinea pig serum as a control, confirmed these findings.

Similar experiments were then carried out with normal serum specimens from rats, chickens, sheep, and humans. In all cases the results of contact of the polymorphonuclear leukocytes of the guinea pig with serums of other species seemed to be: increased viscosity of the cytoplasm (as evidenced by decreased Brownian motion of the cytoplasmic granules), decreased cell diameter, inability to adhere to solid surfaces, increased tendency to clump (over that in guinea pig serum), and a complete absence of motility and pseudopod formation. Degenerative changes ranged from the formation of vesicle-like eruptions at the surface of the cell in rab-

bit serum to a complete separation of the cell wall from the underlying structures and a subsequent clumping of the cell contents at the center of the cell in chicken serum. A sharp decrease in phagocytosis occurred along with the effect on other activities. The results of the tests with rabbit serum are presented in table 4. The results using other serums were very similar with the exceptions noted above.

TABLE 4.—*Activity of guinea pig leukocytes in normal rabbit serum.*

Leukocytes suspended in:	Normal rabbit serum	Normal guinea pig serum	Difference of means / Standard error of difference
Opacity of cytoplasm	3.4*	0.7	22.5
Brownian motion of granules	0.7	3.3	22.5
Pseudopod formation	0.1	1.9	17.0
Motility	0.0	1.5	14.4
Diameter (in microns)	8.7	13.8	29.0
Degenerative changes	1.3	0.2	14.7
Adhesion to slide	0.5	3.2	25.6
Clumping of cells	2.1	0.6	12.2
Percentage phagocytosis	9.1	55.5	9.5

* The figures represent the mean values of 158 measurements.
Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

The inability of serums of other species to support the activity of guinea pig leukocytes might be explained in one of two ways. Either there is some essential substance in guinea pig serum which is absent in the other serums, or there is a substance present in heterologous serums which is harmful for guinea pig leukocytes. The first of these possibilities seemed rather unlikely, as it would be difficult to attribute the rapid onset of degenerative changes to the lack of an essential supporting material. The second possibility was tested by an attempt to discover the presence of harmful substances in rabbit, sheep, human, rat, and chicken serums.

The effect of heat on the damaging action.—The possible presence of a heat labile deleterious factor was investigat d by heating an aliquot portion of a serum sample at 56 C for 30 minutes and then testing the sample and an unheated portion as a control. Such experiments were carried out with each of the different serums which had been tested above. The results of the tests using heated and unheated rabbit serum are presented in table 5. Results with serums of other species were substantially the same. The data presented clearly indicate the presence of a damaging substance, or substances, which may be at least partially destroyed by heating at 56 C for 30 minutes. The difference in activity of the leukocytes in the unheated serum from that in the heated serum was rather general and was reflected in all measurements made in these experiments. Longer heating or heating at slightly higher temperatures failed to increase further the activity of the leukocytes.

TABLE 5.—*Effect of heat on damaging action of rabbit serum.*

Leukocytes suspended in:	Rabbit serum heated at 56 C	Normal rabbit serum	Normal guinea pig serum	Difference of means / Standard error of difference		
	A	B	C	A & B	A & C	B & C
Opacity of cytoplasm	1.1*	3.3	0.4	10.7	4.1	18.0
Brownian motion of granules	3.1	0.6	3.5	12.2	2.3	15.2
Pseudopod formation	1.6	0.1	2.4	9.1	3.2	12.0
Motility	1.2	0.0	2.0	9.9	4.0	12.1
Diameter (in microns)	12.2	8.6	14.5	11.9	7.0	21.8
Degenerative changes	0.6	1.5	0.3	5.3	2.8	8.6
Adhesion to slide	2.5	0.6	3.6	10.5	5.2	18.9
Clumping of cells	1.5	2.0	0.8	2.1	2.5	5.2
Percentage phagocytosis	21.0	10.5	59.5	0.9	3.2	5.0

* The figures represent the mean values of 51 measurements.
Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

Attempts to adsorb deleterious factors

TABLE 6.—*Effect of adsorption with leukocytes on damaging action of rabbit serum.*

Leukocytes suspended in:	Rabbit serum		Normal guinea pig serum	Difference of means		
	Adsorbed	Normal		Standard error of difference		
	A	B	C	A & B	A & C	B & C
Opacity of cytoplasm	2.8*	3.6	1.7	3.3	3.2	6.5
Brownian motion of granules	1.4	0.5	2.3	3.0	2.6	6.2
Pseudopod formation	0.4	0.0	1.2	3.5	3.4	5.6
Motility	0.2	0.0	0.8	2.4	2.9	4.4
Diameter (in microns)	9.8	8.6	12.0	3.9	3.9	6.7
Degenerative changes	1.0	1.1	0.7	1.3	2.9	3.2
Adhesion to slide	1.1	0.4	2.4	3.2	4.6	8.2
Clumping of cells	1.2	1.7	0.6	2.1	3.2	5.0

* The figures represent the mean values of 30 measurements.
Except where indicated, readings were made on the basis of 0 to 4. 4 being the maximum value.

on the surface of guinea pig leukocytes.— An effort was made to remove the damaging substance from rabbit serum by adsorption with a heavy suspension of guinea pig leukocytes. This was accomplished by mixing the serum sample with an equal volume of washed, packed cells and incubating at 2 C with constant, mild agitation for 2 to 4 hours. The degree of removal of the factor was judged by the activity of fresh guinea pig leukocytes in the serums after adsorption as compared to non-adsorbed serum and normal guinea pig controls.

It can be seen from the data in table 6 that some constituent of the serum was adsorbed onto the cells and was thus removed with them when the suspension was centrifuged and the supernatant serum decanted. This indicates that the damaging factor is a substance which can be adsorbed onto the surface of leukocytes and suggests such an adsorption as a possible mechanism for its action. Adsorption at 37 C rather than at 2 C gave essentially the same results, and similar experiments with serum of other species also showed the same effect. Attempts to remove more of the material by repeated adsorption with fresh cell suspensions, allowing each suspension to remain in contact with the serum for 10 hours at 2 C, were unsuccessful. Furthermore, the damaging effect of the treated serum was as great as that of the untreated, suggesting that on prolonged contact elution of the adsorbed material occurs.

Irreversibility of deleterious effects.— To determine whether the deleterious effects of the serums were permanent or reversible and whether they were due to the presence of an adsorbed substance on the cell surface, the leukocytes were suspended first in the serum to be in-

TABLE 7.—*Effect of previous contact with rabbit serum upon the activity of guinea pig leukocytes in homologous serum.*

Leukocytes suspended in:	Normal rabbit serum	Guinea pig serum After suspension in:		Normal guinea pig serum	Difference of means				
		Rabbit serum	Guinea pig serum		Standard error of difference				
	A	B	C	D	A & B	A & D	C & D	B & D	B & C
Opacity of cytoplasm	3.7*	3.4	1.0	0.7	0.8	9.0	0.5	6.2	3.8
Brownian motion of granules	0.4	0.7	3.1	3.5	0.8	8.9	0.8	6.1	4.7
Pseudopod formation	0.0	0.0	1.3	1.8	0.0	5.4	0.9	5.4	2.6
Motility	0.0	0.0	0.9	1.2	0.0	4.6	0.6	4.6	1.5
Diameter (in microns)	8.5	8.8	12.5	14.0	0.8	14.4	1.9	12.3	4.6
Degenerative changes	1.1	1.5	0.3	0.1	1.1	5.6	0.8	3.7	3.2
Adhesion to slide	0.5	0.5	2.6	3.3	0.0	8.4	1.3	8.4	3.7

* The figures represent the mean values of 10 measurements.
Except where indicated, readings were made on the basis of 0 to 4. 4 being the maximum value.

vestigated, incubated for 15 to 30 minutes, carefully separated by centrifugation, washed twice with normal guinea pig serum, and finally resuspended in normal guinea pig serum. The purpose of this procedure was to remove any substances adhering to the cell surface and thus indicate permanent damage by comparison of the activity of these cells with that of fresh normal leukocytes in normal guinea pig serum. A further control was the activity of untreated cells in the various harmful serums. Table 7 summarizes these experiments and shows that the damaging action is permanent. No significant change in activity occurred when the cells were washed free of heterologous serum and resuspended in normal guinea pig serum. Cells suspended first in guinea pig serum and then carried through the washing procedure showed no evidence of decreased activity.

Effect of dilution with guinea pig serum.—Finally, experiments were undertaken to determine the effect of dilution on the damaging action of the different serums. Fresh normal guinea pig serum was added to the heterologous serums in the following ratios: 1:1, 1:2, 1:4, and 1:100. The results are presented in table 8 and show a rather marked reduction in deleterious effects when the rabbit serum was mixed with an equal volume of normal guinea pig serum. A somewhat lesser degree of reduction occurred when one volume of guinea pig serum was added to two volumes of rabbit serum, and no effect was noted with smaller amounts.

TABLE 8.—*Effect of dilution on damaging action of rabbit serum.*

Leukocytes suspended in:	Guinea pig serum: rabbit serum				Normal serum		Difference of means			
	1:1	1:2	1:4	1:100	Rabbit	Guinea pig	Standard error of difference			
	A	B	C	D	E	F	A & E	A & F	C & E	C & F
Opacity of cytoplasm	1.4*	2.0	2.7	3.9	3.6	0.0	4.9	3.9	1.8	6.5
Brownian motion of granules	2.8	2.0	0.3	0.3	0.5	4.0	4.5	3.3	0.3	9.0
Pseudopod formation	0.9	0.0	0.0	0.0	0.0	1.7	6.6	2.5	0.0	5.5
Motility	0.4	0.0	0.0	0.0	0.0	1.7	1.3	3.2	0.0	5.5
Diameter (in microns)	11.6	10.0	8.3	9.1	8.5	14.7	3.6	4.4	0.3	14.0
Degenerative changes	0.8	1.0	1.3	0.8	1.3	0.0	1.9	4.0	0.2	3.3
Adhesion to slide	1.9	1.3	0.3	0.3	0.3	3.4	4.6	4.4	0.2	6.3
Clumping of cells	1.5	2.3	2.7		2.4	0.6	2.2	2.5	0.6	4.3

* The figures represent the mean values of 8 measurements.
Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

Comparison of the effect of serum from the same and different guinea pigs on the activity of guinea pig leukocytes

The presence in the serums of a number of species of substances harmful to guinea pig leukocytes suggested an investigation of compatibility within the species. To answer this question, samples of serums from a number of guinea pigs were tested individually against the polymorphonuclear leukocytes of several guinea pigs. In this manner it was possible to compare the activity of the leukocytes from guinea pig A when suspended in serum from guinea pig A, B, C, D, etc. Data from such studies are presented in table 9. It will be seen that the values are identical within the limits of error of the technique used, thus eliminating the possibility of differences in effect of various guinea pig serums on guinea pig leukocytes, at least as far as could be detected by this method.

The importance of ascorbic acid

An interesting observation frequently noted during these studies was the varia-

tion in activity of leukocytes from different guinea pigs even when suspended in the same serum sample. This variability seemed to bear no relationship to the serum but was a reflection of differences in the leukocytes themselves. The work of Nungester and Ames[3]

TABLE 9.—*Effect of serum from one guinea pig upon the leukocytes of another guinea pig.*

Leukocytes suspended in:	Serum from guinea pig		Difference of means
	Another	Same	Standard error of difference
Opacity of cytoplasm	0.4*	0.2	0.9
Brownian motion of granules	3.5	3.4	0.3
Pseudopod formation	1.8	1.8	0.1
Motility	1.2	1.2	0.0
Diameter (in microns)	14.2	14.7	0.9
Degenerative changes	0.0	0.0	0.3
Adhesion to slide	3.6	3.8	0.9

* The figures represent the mean values of 35 measurements.
Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.

The results showed a significant increase in the activities of the cells as the vitamin C content approached normal levels (0.31 mg percent or higher). The measurements which showed significant differences were cell diameter, psuedopod formation, motility, and phagocytosis. No further increase in activity was observed when the ascorbic acid levels were increased even to 4 to 5 times the normal concentration.

DISCUSSION

The combined use of darkfield illumination and a warm box technique permitted the observation of several manifestations of activity by leukocytes under a variety of exeprimental conditions. In this type of preparation, intensity of Brownian motion by the granular inclusions served as a rough index to the viscosity of the cytoplasm. Changes in the opacity of the protoplasm rather closely paralleled the extent of Brownian motion of the granules and likewise seemed to reflect viscosity alterations. Studies on amebae[4] indicate that in these cells the mechanism of pseudopod formation and ameboid motion depends upon the ability of cells to convert their protoplasm rapidly

TABLE 10.—*Effect of ascorbic acid concentration in exudates upon the activity of leukocytes from these exudates.*

	Ascorbic acid levels of exudates—mg percent						Difference of means			
	0.0–0.3	0.31–0.60	0.61–0.90	0.91–1.20	1.21+	*All*	Standard error of difference			
	A	B	C	D	E	F	A & B	B & C	A & F	B & F
Opacity of cytoplasm	0.9*	0.8	0.7	0.7	0.6	0.7	0.2	0.4	0.6	0.7
Brownian motion of granules	2.9	3.4	3.3	3.3	3.4	3.3	1.4	0.3	1.3	0.4
Pseudopod formation	1.4	2.1	1.8	1.6	2.0	2.0	2.1	1.0	2.0	0.7
Motility	1.0	1.7	1.5	1.5	1.7	1.6	2.6	0.8	2.8	0.6
Diameter (in microns)	11.9	13.9	14.1	13.9	13.6	13.8	3.0	0.6	3.0	0.4
Degenerative changes	0.4	0.2	0.1	0.2	0.3	0.2	1.0	1.0	1.0	0.0
Adhesion to slide	3.1	3.4	3.4	3.2	3.5	3.4	0.6	0.0	0.5	0.2
Clumping of cells	0.3	0.5†					0.0			
Percentage phagocytosis	23.3	49.6†					5.7			

* These figures represent the mean values of 291 measurements.
Except where indicated, readings were made on the basis of 0 to 4, 4 being the maximum value.
† These figures include all exudates with ascorbic acid values higher than 0.31 mg percent.

has shown that the ascorbic acid content of the donor animal and the phagocytic activity of the leukocytes are directly related. In the work reported here, ascorbic acid assays were made routinely* on the exudates, and an attempt was made to correlate the ascorbic acid values of the exudates with the activity of the leukocytes obtained from them. The data are presented in table 10.

* Mr. D. Alonso is to be thanked for determining the values.

4. Mast, S. O. 1931, Locomotion in *Amoeba proteus*. Protoplasma, **14**: 321.

from a sol to a gel and vice versa. It has been shown further that these changes may be stimulated by a variety of inorganic ions and organic materials which are capable of penetrating the cell wall. Thus pseudopod formation and motility are correlated with changes in viscosity of the cytoplasm. This work has been applied to leukocytes by analogy.[5]

Ameboid motion of leukocytes is also dependent upon the ability of the cells to spread out at the interface between the suspending fluid and a solid surface, such as a glass slide, and to become adherent to the solid material. When they are free floating, white blood cells tend to assume a spherical form. Changes from the spherical to the flattened form (which exists when the cells spread out at an interface) occur without any apparent change in volume. Hence, the cell diameter is a rather good criterion of spreading, if no pronounced osmotic changes have occurred. Measurement of the diameter of leukocytes, observations of their adhesion to the slide and coverslip, and evidence of pseudopod formation and ameboid motion all serve as indicators of reaction at the cell surface, in contrast to the evidences of viscosity alteration, which probably reflect penetration of the cell wall by soluble materials. In these studies the ability to phagocytize has been closely associated with spreading, pseudopod formation, and motility, and therefore is possibly related to changes at the cell surface as well as internal changes.

Though many investigators have reported normal cell activity by leukocytes suspended in a physiologically balanced salt solution, we have been unable to confirm these findings. In all instances there was a marked decrease in activity of cells suspended in Locke solution from that which occurred when the leukocytes were suspended in serum. Furthermore, a careful check of the literature showed that in almost every instance where normal activity in salt solutions was claimed, the leukocytes were obtained from whole blood and sufficient care was not taken to insure complete removal of serum from the cell suspension. This, along with the failure to confirm the findings of Friedemann and Schönfeld[1] concerning the importance of viscosity and those of Delaunay and Pages[2] on the role of complement in leukocyte motion, emphasizes the complexity of problems involved in studying the effect of serum on living cells.

The demonstration of an adverse effect of specific antileukocyte serum (prepared in the rabbit) upon the polymorphonuclear leukocytes of the guinea pig, and the subsequent demonstration of a very similar effect of normal rabbit serum, led to an investigation of the effect of other heterologous serums upon the white blood cells of this species. During the period from 1907 to 1916 many workers[6–12] observed the presence

5. DeBruyn, P. P. H. 1946, The ameboid movement of the mammalian leucocyte in tissue culture. Anat. Rec. **95**: 177.

6. Bächer, St. 1907, Über Beeinflussung der Phagozytose durch normales Serum, Ztschr. f. Hyg. **56**: 33.
7. Reudiger, G. F. and Davis, D. J. 1907, Phagocytosis and opsonins in the lower animals. J. Infect. Dis. **4**: 333.
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9. Porges, O. 1909, Ueber Opsonine für Stärke. Ztschr. f Immunitäts forsch u. exper. Therap. **2**: 4.
10. Ruchlädew, N. 1910, Untersuchungen zur Kritik der Methodik Chemotactischer Versuche und zur Biologie der Leukozyten. Ztschr. f. Biol. **54**: 533.
11. Löhner, L. 1915, Über Normalleukotoxine und ihre Beziehungen zur Phagozytose und Blutsverwandtschaft. Pflüger's Arch. f. d. ges. Physiol. **162**: 19.
12. Keitler, H. and Wolfsgruber, R. 1916, Ueber Beeinflussung der Phagozytose bei Pferdeleukozyten durch menschlichen Serum. Wien. klin. Wchnschr. **29**: 1429.

of "leukocytotoxins" in heterologous serums, after which time the subject received little attention. The most frequently observed effects of the "leukocytotoxins" was inhibition of phagocytosis and degenerative cell changes, both observed in stained smears.

In the present work, rabbit, rat, sheep, human, and chicken serums were found to damage guinea pig leukocytes in that order, chicken serum being the most effective. The presence of a substance or substances in the heterologous serums which were responsible for the deleterious effects seemed rather evident in view of the fact that damage to the cells was irreversible and exposure for even a brief interval resulted in permanent changes. This view was substantiated by the finding that the ability of such serum to damage leukocytes was markedly diminished if the serums were heated at 56 C for 30 minutes prior to the addition of the cells. It was further strengthened by the demonstration of a reduction in harmful effect when the serums were previously adsorbed with guinea pig leukocytes.

The partial elimination of the damaging action by heat and the demonstration that it could be removed by diluting the heterologous serums with an equal volume of guinea pig serum suggested a possible relationship to complement. This, however, appeared unlikely when the species distribution was examined. Species distribution also eliminated Forssman antibodies as suspects in this connection.

Failure to remove all the damaging action of heterologous serums either by heating at 56 C or by adsorption techniques suggests the possibility of there being two or more substances present in such serums which are responsible for effects noted.

Results using heterologous serums very naturally brought to mind the question of compatibility within the species. Tests made using cells from one guinea pig with serum from the same and other guinea pigs failed to show any differences in the ability to support leukocyte activity. The results of these experiments were very clean cut.

The relationship of ascorbic acid nutrition in the donor animals to the activity of the leukocytes which they produced was very interesting. As previously noted, cells from exudates which had vitamin C assay values of 0.31 mg percent or higher were quite active, and increased concentrations of ascorbic acid failed to give cells with greater activity. On the other hand, leukocytes from exudates with a level of vitamin C below 0.30 mg percent were definitely less active. Blood ascorbic acid values were run in each instance, and while blood levels and exudate levels were not identical, there was a marked parallelism between the two.

Pseudopod formation, motility, and phagocytosis were the measurements affected by variation in ascorbic acid concentration. The fact that these activities increased until normal levels were attained and then showed no further change would seem to suggest that vitamin C may play a part in initiating these activities but cannot cause an increase once they have begun. The exact mechanism of action, however, is not known. The work of Reid[13] suggested that the action might take place at the cell surface and be tied up with the release of calcium for intracellular use. This was supported by Ames et al,[14] who found at the cell surface a concentration of a material which strongly reduced silver nitrate.

13. Reid, M. E. 1943, Interrelationships of calcium and ascorbic acid to cell surfaces and intracellular substances and to physiological action. Physiol. Rev. **23**: 76.
14. Ames, Ada M., Alonso, D. and Nungester, W. J. 1948, The correlation between the ascorbic acid content of tissues and phagocytosis. Proc. Soc. Am. Bacteriologists, **92**.

SUMMARY

1. A study has been made of the effect of serum on the activity of the polymorphonuclear leukocytes of the guinea pig using a warm stage darkfield technique. Measurements were made of the opacity of the cytoplasm, Brownian motion of the granules, pseudopod formation, ameboid motion, cell diameter, degenerative changes, adhesion to solid surfaces, clumping and phagocytosis of leukocytes. It combines several well known techniques to make possible the observation of these phenomena in a single preparation under carefully controlled conditions.

2. Homologous serum was essential for the normal activity of the polymorphonuclear leukocytes of the guinea pig. Replacement of the serum with a balanced salt solution did not furnish optimum conditions. Adjustment of the viscosity of these solutions to that of serum by the addition of gum arabic also failed to provide the necessary conditions for normal leukocyte activity. The presence or absence of complement played no role.

3. Serums of the rabbit, rat, sheep, chicken, and human contained substances which were deleterious to the polymorphonuclear leukocytes of the guinea pig. This harmfulness was evidenced by a loss of pseudopod formation, motility, and phagocytosis. The cells were no longer able to adhere to glass surfaces. They tended to become spherical and opaque and to show degenerative changes. The effects were not reversible but could be partially destroyed by heating at 56 C for 30 minutes. Adsorption with guinea pig leukocytes removed some of the deleterious action. The ability of rabbit serum to reduce leukocyte activity disappeared upon dilution with guinea pig serum, 1:1 dilution being most effective.

4. Serum from other guinea pigs was just as effective in supporting the activity of a given leukocyte preparation as was serum from the donor of the cells.

5. A definite level of ascorbic acid in the exudate was required for the initiation of pseudopod formation, motility, and phagocytosis. Increased amounts of ascorbic acid did not increase these activities.

THE SULFONAMIDES AS FACTORS IN INCREASING SUSCEPTIBILITY TO PARASITIC INVASION

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There is a massive literature on the phenomena of acquired immunity, but there appears to be little known concerning the factors which govern the innate immunity of organisms to infectious agents, other than the negative information that cellular or antibody factors seem to play little or no part. In this paper the expression "innate immunity" is used to indicate the initial, inherent or constitutional degree of susceptibility or resistance of a host animal to a specific parasitic agent.

The malarial-infected mosquito is particularly well adapted for studies designed to determine the probable role of specific biochemical factors in the host-parasite relation. With this host, it is possible to dissect out the stomach, and by counting all the oöcysts present on the stomach wall, to determine exactly the total parasite population of each individual mosquito. This precise measure of the intensity of the infection thus makes it possible to evaluate experimental differences in parasite population between individual mosquitoes and groups of mosquitoes on a strictly quantitative basis. Furthermore, methods have already been devised for administering specific chemical or biochemical compounds in the mosquito diet and maintaining small mosquito colonies on these regimens for varying periods of time.[1,2] The present studies were undertaken to determine whether specific biochemical compounds, thus administered, could be shown to affect the susceptibility of the mosquito *Aedes aegypti* to the avian malarial parasite *Plasmodium gallinaceum*.

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1. Terzian, L. A. 1947, A method for screening antimalarial compounds in the mosquito host. Science, **106**: 449.
2. Terzian, L. A., Stahler, N. and Weathersby, A. B. 1949, The action of antimalarial drugs in mosquitoes infected with *Plasmodium gallinaceum*. J. Infect. Dis. **84**: 47–55.

MATERIALS AND METHODS

In these experiments, laboratory strains of *A. aegypti* were infected with a strain of *P. gallinaceum* designated as the 8A strain. The compounds to be evaluated were prepared in solutions calculated in terms of the salt, and were added in various concentrations to a 4 % sugar nutrient solution used as the standard maintenance diet for infected mosquitoes. There was no mortality in either the control or the experimental groups during the time required to complete an experiment.

The mosquitoes were set up in cylinders in groups of 50 females, and for each experiment, mosquitoes of the same age, derived from the same larval colony, were used. In all the experiments, sugar and drug solutions were made available to the adult mosquitoes 24 hours after emergence, and the mosquitoes were then maintained on these solutions for the next 72 hours in order to allow them to build up the drug level prior to their exposure to the infective blood meal. At the end of the 72-hour period the mosquitoes were then exposed to a chick infected with *P. gallinaceum*. Feeding was usually completed within a matter of 10 minutes, following which all the unfed females were culled out of the cylinders. Meanwhile, drug administration was continued until the completion of an experiment.

It was found that when a series of mosquito groups were fed consecutively, even though on the same chick, there were, in some cases, significant differences in the oöcyst counts of each group, unrelated to any experimental procedure or diet. For example, if 4 cylinders were infected in consecutive order on the same bird, there could be a significant difference between the oöcyst counts of the mosquitoes from cylinder 1 and the oöcyst counts of the mosquitoes from cylinder 4, even though the total time elapsed between the first and last feeds inclusive might

not exceed one hour. For this reason, each group or cylinder of mosquitoes maintained on a specific drug diet was paired with its control group, maintained on sugar alone, and both groups were fed simultaneously on the same bird. This procedure eliminated the possibility of error due to a lapse in time between feedings of individual groups.

In evaluating the intensity of the parasitemia in the mosquito the procedure was to count the total number of oöcysts present on the entire stomach at a magnification of approximately 300 diameters. Since the amount of blood taken up by the mosquito determines the parasite dosage, and this could affect the total number of oöcysts present on the stomach wall, it was necessary to control such variation by producing adult mosquitoes of nearly uniform size. Weight measurements of such mosquitoes, made both before and after feeding, showed no significant differences between individuals, and between control and experimental groups, either in the prefeeding weights of the mosquitoes or in the calculated weights of the blood meals In general, oöcyst counts were done on the 5th, 6th, 7th and 8th day of the infection, the day of the infective blood meal being designated as day 0. The control groups were usually counted on days 5 and 6, and since some of the drugs subsequently inhibited parasite development the experimental groups were counted on days 7 and 8. In those cases in which there was no subsequent inhibition of the parasite all counts were completed by day 7, since by day 8 the oöcysts begin to rupture. To eliminate any possibility of error due to counts being made on different days of the parasite cycle, in many experiments oöcyst counts of the control groups and the experimental groups were both made on the same day.

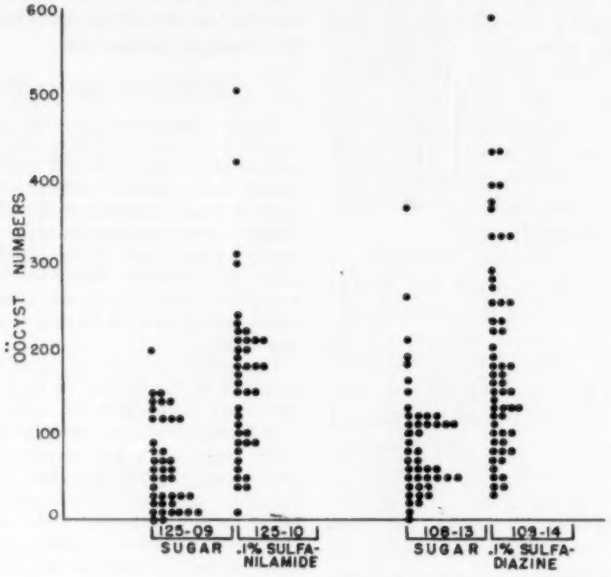

FIG. 1.—A comparison of the oöcyst densities of mosquitoes infected with *P. gallinaceum* maintained on diets of 4% sugar solutions as compared to infected mosquitoes maintained on sugar with 0.1% sulfanilamide added and on sugar with 0.1% sulfadiazine added.

RESULTS AND COMMENTS

In figure 1 each point represents an individual mosquito, while the level of infection or the total number of oöcysts present on the stomach wall of each is shown by the scale on the left of the figure. The total number of points within a column represents the total number of

mosquitoes within a group, since in each experiment all the mosquitoes within a group were dissected and the total number of oöcysts on the stomach wall of each determined. From the data presented in these two representative experiments, it is evident that both sulfanilamide and sulfadiazine, administered in the mosquito diet in a 0.1 % concentration, have increased the susceptibility of the mosquito *A. aegypti* to the avian malarial parasite *P. gallinaceum*. This fact is evidenced by the significantly higher oöcyst densities in each of the experimental groups shown as compared to their paired control groups. Although there is considerable variation in the oöcyst counts of the various individuals within a given group, nevertheless, there is a specific, statistical consistency about the distribution in the counts within a group of this size, for it has been found that increasing the number of individuals within a group, to numbers higher than those in the experiments cited, does not significantly affect the relative distribution according to oöcyst density. Examination of the data for experiments 125 and 108, presented in figure 1, shows that in cylinder 125–10, maintained on 0.1 % sulfanilamide, and again in cylinder 108–14, maintained on 0.1 % sodium sulfadiazine, there is a more than considerable shift upward in the oöcyst densities of the mosquitoes in these groups, as compared to the individuals in their respective paired control cylinders, 125–09 and 108–13. For example, in experiment 125, in the control group 125–09, there were only 3 mosquitoes with counts above 150 oöcysts per mosquito, the highest single count reaching 200 oöcysts. On the other hand, in experimental cylinder 125–10, there were 24 mosquitoes with counts of 150 oöcysts or higher, and in this case the highest single count soared to 505 oöcysts. Although it would appear as if the increase in susceptibility is more pronounced in those individuals which have a comparatively lesser resistance to the parasite, nevertheless the effect is equally marked in those individuals which apparently have an inherently greater resistance. Thus, there were in control cylinder 125–09, 17 mosquitoes with counts below 50 oöcysts, while in experimental cylinder 125–10 there were only 3 individuals with counts below 50 oöcysts. Similarly, in experiment 108 there is the same significant shift upward in the oöcyst densities of the individuals in the experimental group 108–14 as compared to its paired control group 108–13. In cylinder 108–13 there were only 3 individuals with counts of 200 or more oöcysts, with 360 oöcysts as the single highest count, while in group 108–14 there were 21 mosquitoes with counts of 200 oöcysts or higher, the highest single count reaching 605 oöcysts. And again, there is evident the same upward shift in oöcyst counts even among the more resistant individuals. These differences, when tested in a three by two chi square table, show P to be less than 0.01 for both experiment 125 and experiment 108. Similar tests were used for all the experiments cited and constitute the basis for the statement of P values. The P values for all experiments cited are summarized in table 1.

It is important to note that this specific effect of increasing the susceptibility of the mosquito host, *A. aegypti*, to the avian malarial parasite, *P. gallinaceum*, requires a specific optimal drug concentration. This is shown by the data presented in figure 2. Again, as indicated by experiments 123 and 124, in the groups maintained on an 0.1 % drug concentration, there is a marked increase in susceptibility to the parasite as evidenced by the significant increase

TABLE 1.—*The chi square and probability values for the experiments cited in the text.*

Infection source bird no.	Experiment no.	Group no. and regimen		Chi square	Probability
1952	125	09(Sugar)	vs 10(0.1 S-amide)	18.093	<0.01
523	108	13(Sugar)	vs 14(0.1 S-diazine)	14.900	<0.01
976	123	13(Sugar)	vs 16(Sugar)	0.9576	0.50–0.70
		13(Sugar)	vs 14(0.1 S-amide)	9.539	<0.01
		16(Sugar)	vs 15(0.2 S-amide)	8.901	0.01–0.02
		14(0.1 S-amide)	vs 15(0.2 S-amide)	10.0398	<0.01
1117	124	09(Sugar)	vs 12(Sugar)	2.447	0.20–0.30
		09(Sugar)	vs 10(0.1 S-diazine)	7.132	0.02–0.05
		12(Sugar)	vs 11(0.2 S-diazine)	1.029	0.50–0.70
		10(0.1 S-diazine)	vs 11(0.2 S-diazine)	4.454	0.10–0.20
878	136	13(Sugar)	vs 18(Sugar)	0.554	0.70–0.80
		13(Sugar)	vs 14(0.1 S-diazine)	10.399	<0.01
		18(Sugar)	vs 17(0.1 S-diazine plus 0.2 PABA)	0.000	>0.95
		14(0.1 S-diazine)	vs 17(0.1 S-diazine plus 0.2 PABA)	10.734	<0.01

The author wishes to express his sincere appreciation to Dr. E. A. Jerome, who was responsible for all the statistical analyses in this work.

in oöcyst numbers. The differences between cylinder 123–14, maintained on sulfanilamide, and its paired control 123–13, are significant at the 1 % level; and the differences between cylinder 124–10, maintained on sulfadiazine, and its paired control 124–09, are significant at the 5% level. With either

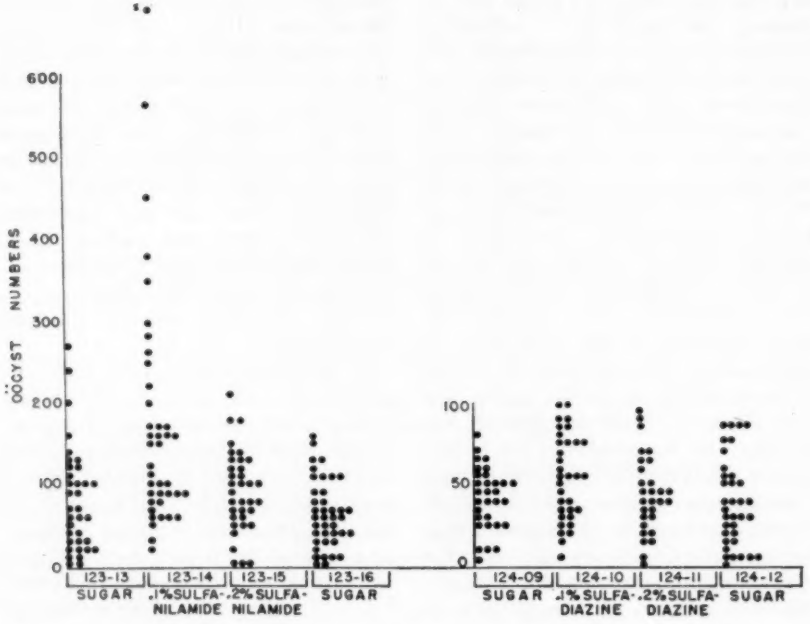

FIG. 2.—A comparison of the oöcyst densities of infected mosquitoes maintained on sugar and of mosquitoes maintained on sugar with 0.1% and 0.2% added sulfanilamide and sulfadiazine.

drug, however, at a concentration of 0.2% the effect of the drug is considerably decreased. The differences between group 123–15, maintained on 0.2% sulfanilamide, and its paired control cylinder 123–16 are less significant; and the differences between cylinder 124–11, maintained on 0.2% sulfadiazine, and its control cylinder 124–12 are not at all significant. Thus, in both experiments there is a consistent and obvious decline in oöcyst numbers in the groups maintained on an 0.2% drug concentration as compared to the mosquitoes maintained on an 0.1% concentration. It should be noted that in each separate experiment cited in this study all the mosquitoes were fed on the same infected chick. For example, control cylinder 123–13 and its experimental pair, 123–14 were fed simultaneously on bird 976, and then control cylinder 123–16 and its experimental pair, 123–15, were fed about 15 minutes later on the same bird. This fact, that all the mosquitoes in a specific experiment received their infective meals from the same chick, provides a basis for comparison between the two control groups in each experiment. This comparison makes even more evident the fact that in the two experimental groups maintained on the 0.2% concentration of sulfanilamide and sulfadiazine the effect on the innate susceptibility of the mosquitoes to the parasite has declined. The reasons for this evident loss in drug activity at higher concentrations are not clear as yet and require additional investigation.

In a representative experiment cited in figure 3, evidence is presented to show that in increasing the susceptibility of this host to the infectious agent, sulfadiazine is acting through a mechanism related to the para-aminobenzoic acid metabolism of the host. Again, based upon the frequency distribution according to oöcyst numbers there is evident a significantly increased susceptibility to infection in the mosquitoes in group 136–14, maintained on an 0.1% concentration of sulfadiazine, as compared to the mosquitoes in the paired control group 136–13, maintained on sugar alone. On the other hand, there is no difference in the relative frequency according to oöcyst numbers between the mosquitoes of group 136–17, maintained on a regimen of 0.1% sulfadiazine plus 0.2% para-aminobenzoic acid, and its paired control group 136–18, maintained on sugar, so that each group resembles the other, and both differ to the same extent from group 136–14. Thus, it is evident that para-aminobenzoic acid, administered in the mosquito diet in a concentration of 0.2 % along with the 0.1% sulfadiazine, completely inhibits the action of sulfadiazine and nullifies its effects on the susceptibility of the host.

The fact that in increasing the susceptibility of *A. aegypti* to P. *gallinaceum*, sulfadiazine is acting through its relation to para-aminobenzoic acid metabolism and the fact that it has already been shown that para-aminobenzoic acid is essential to the economy of the parasite during a specific stage in its developmental cycle in the invertebrate host[2] lends support to the assumption that in increasing host susceptibility to the parasite, the sulfonamides are acting directly on the host. There is little reason to believe that the lack of para-aminobenzoic acid could at one time increase the invasive powers or the virulence of the parasite when at another time this lack completely arrests its further development. It is not unlikely, therefore, that the sulfonamides, by depriving the host of its available para-aminobenzoic acid, disturb or remove a factor essential to the innate immunity mechanism of the mosquito, thus affecting the inherent equilibrium in this host-parasite relation.

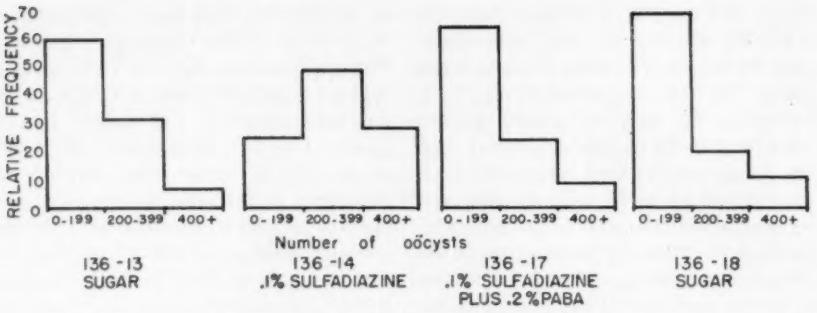

FIG. 3.—A comparison of the oöcyst densities of mosquitoes maintained on 0.1% sulfadiazine and of mosquitoes maintained on 0.1% sulfadiazine with added 0.2% para-aminobenzoic acid.

SUMMARY AND CONCLUSIONS

1. It has been demonstrated quantitatively that a specific biochemical compound can influence the innate immunity of a host to a specific parasite. Thus, it has been shown that sulfanilamide and sulfadiazine, administered in 0.1% concentration in the mosquito diet, will increase the innate susceptibility of *Aedes aegypti* to *Plasmodium gallinaceum*.

2. The maximal effect of these sulfonamides depends upon a specific optimal drug concentration, and the effect is decreased when sulfanilamide and sulfadiazine are administered in 0.2% concentration in the mosquito diet.

3. Para-aminobenzoic acid, administered in sufficient concentration along with effective concentrations of sulfadiazine, inhibits the action of sulfadiazine and nullifies its effects on the susceptibility of the host.

4. This relation of metabolite to innate immunity may well be fundamental to the mechanism of innate immunity in animals in general, in which case it may provide in part an explanation for the nature of the mechanisms of innate immunity and of host-parasite specificity.

THE PLACENTAL TRANSMISSION OF ANTIBODIES AND SERUM γ GLOBULINS

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As the maternal and fetal blood course through independent channels, antibodies, as serum proteins, must encounter the placental barrier before their entrance into the fetal circulation can be accomplished. Branches of the umbilical artery and vein of the fetus terminate in villi surrounded by pools of maternal blood within the uterus, and it is through these that interchange of constituents of the two circulations takes place.

The passage of substances from the maternal blood to the fetus may occur by several different processes:[1,2] simple diffusion, osmosis, selective absorption, activity of the chorionic epithelium, and probably other ill-understood mechanisms.

Many substances have been demonstrated to pass the placental barrier, but the fate of the plasma protein molecule is not clear. Plasma proteins have been found to be more concentrated on the maternal than the fetal side of the circulation.[3] Considering their large molecular size, it has been felt that albumin and globulin probably do not pass through the placenta but are synthesized from their constituent amino acids by the fetal tissues.[2]

Though pathogenic bacteria,[1,2] trypanosomes, and the malarial protozoan do not usually pass through the uninjured placenta, spirochetes and viruses frequently do. The placental transmission of serum antibodies in the human, rabbit, and guinea pig has been extensively studied and is accepted in these species, although this transfer does not occur in the goat, sheep, cow, or horse.[4] Among those antibodies studied and found to be transmitted from the mother to the fetus in utero are:[1,2,4] antitoxins against diphtheria and tetanus, typhoid agglutinins, bacteriolysins, complement fixing antibodies, agglutinins, hemolysins, precipitins, protein sensitizing antibodies, and isoagglutinins determining blood groups. Immune bodies against certain virus infections may likewise be transmitted through the placenta.[5] Blocking antibodies against pollen antigen evidently permeate the human placental membrane,[6] although the skin sensitizing antibody or reagin is rarely transmitted to the fetus.[6,7]

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Recent studies[8–19] all point to the fact that antibodies are modified serum globulins and may be identical with or similar to the serum γ globulin fractions. Ascoli[20] found that if large amounts of egg, cow, or horse serum were injected subcutaneously in pregnant rabbits, these proteins were found by precipitin tests in the serum of the fetus removed by Cesarean section one hour to several days later. However, if the heterologous protein was given orally or injected subcutaneously in small amounts then the fetal serum did not demonstrate the precipitin reaction to these proteins. Holford[21] injected pregnant rabbits with crystalline egg albumin 5 days before term and found this protein in the serum of the newborn immediately after birth. Ratner, Jackson, and Gruehl[4] have been able to demonstrate transmission of protein hypersensitivity from pregnant guinea pigs to their offspring.

This report presents additional evidence on the placental transmission of serum γ globulins and antibodies in the pregnant rabbit.

MATERIALS AND METHODS

The placental transmission of normal serum γ globulins and homologous antiserum in pregnant rabbits was studied noting whether or not these substances were transmitted through the placenta, their time of appearance in the fetal circulation, the relative titers in the maternal and fetal circulation, and whether or not any differences in the transmission of normal serum globulin and antibodies could be detected.

A series of pregnant female rabbits, near term, were given intravenous injections of either 300 mg of bovine serum γ globulin (Fraction II, Armour), 150 or 300 mg normal human serum γ globulin (Lederle), or 10 cc of rabbit antibovine or antihuman γ globulin serum of approximately

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20. Ascoli, cited by Ratner, B., Jackson, H. C. and Gruehl, H. L.[4]
21. (a) Holford, cited by Ratner, B., Jackson, H. C. and Gruehl, H. L.,[4] (b) Holford, F. E. 1926, Placental transmission of foreign protein. J. Bact. **11**: 106.

the same titer. At varying time intervals ranging from 1 hour to 120 hours after receiving the intravenous injection, termination of pregnancy by Cesarean section was done under ether anesthesia on one rabbit of each series. The fetuses were removed from the uterus and the umbilical cord clamped and severed from the placenta. The fetuses were then washed free of maternal blood and bled from the umbilical cord, the heart, the great vessels severed at the base of the heart, and the jugular and neck vessels. In those instances where the amount of serum from the individual fetus was too small in quantity, it was necessary to pool serums of 2 to 3 fetuses for study. Immediately prior or during the removal of the fetuses from the uterus, maternal blood was collected either from the central artery of the ear or by cardiac puncture. Placental blood was collected by placing the placenta in a small beaker, cutting it into small pieces, and allowing the blood to drain off. Serums from maternal, fetal, and placental blood in any one series of animals receiving the same substance were studied by exactly the same technique.

Preparation of antiserum.—Healthy adult albino male rabbits of the New Zealand strain were immunized with heterologous serum γ globulin according to one of the following schedules: (1) human serum γ globulin (Lederle), 5 mg intravenously 4 times a week for 3 weeks; (2) bovine serum γ globulins (Fraction II, Armour) that had been alum precipitated to give a concentration of 1.5 mg of globulin per ml in physiological saline, 16 injections, 4 per week consisting of 3 injections of 1 ml, 3 of 1.5 ml, 4 of 2 ml, 4 of 3 ml, and 2 injections of 5 ml. Seven days following the final injection the animals were bled by cardiac puncture. The serum of each animal was titrated and those of like titer were pooled.

Analysis of serum content.—Qualitative tests for homologous serum globulin were made by the "ring test" method. Rabbit antiserum (bovine or human γ globulin) with an antibody titer of 1:640 and capable of detecting 1 part of antigen per million was placed in a 7 mm tube. By means of a capillary pipette the serum to be tested (maternal, placental, or fetal), to which had been added a minimal amount of physiological saline, was carefully layered on top of the rabbit antiserum. A ring of precipitate at the junction of the two layers after one-half hour indicated a positive test denoting the presence of the species specific serum γ globulin. Where an attempt was being made to detect the presence of antibodies in serum, a saline solution of either human or bovine γ globulin 1:10, 1:100, or 1:1000 was layered on top of the unknown serum being tested. Those serums that showed the presence of the homologous γ globulin or antibody for which the tests were made, were then further examined for antigen or antibody titers respectively, by precipitin tests. Antibody titer was determined by adding 1 cc of increasing double dilutions of serum in saline to 1 cc of antigen, 1:5000. The tubes were incubated for one hour in a water bath at 37 C, then placed in the refrigerator. Readings were made at the end of 24 and 48 hours. The antibody titer was read as the weakest dilution of serum that showed the presence of a precipitate. Similar titrations for heterologous γ globulin content of serum were made by varying the saline dilution of the unknown serum and keeping the specific anitserum constant with which the unknown serum was mixed and incubated. Control tests using normal rabbit serum and physiological saline were run simultaneously.

RESULTS

A series of 32 pregnant rabbits received heterologous serum γ globulin intravenously, 19 received human γ globulin (3 were given 150 mg and 16 received 300 mg), and 13 received 300 mg of bovine γ globulin. Studies on the maternal, placental, and fetal serums revealed that both the human and bovine serum γ globulins were transmitted through the rabbit placenta and that passage of both is essentially similar in all respects. The results of these findings are summarized in table 1. Control studies on the maternal serum prior to administration of the protein showed the absence of any substance in the blood stream that might mask the test, and thus all serum titers reported are true measurements. Between one and four hours following the intravenous administration of the γ globulin there was a marked and rapid drop in the titer of maternal serum. In those animals that had received human γ globulin the titer after one hour of circulation was 1:1500; after 2 hours 1:500; at 4 hours 1:100; and at 6 hours 1:80. Following this there was a slow gradual decline in titer in those animals studied during the

TABLE 1.—*Heterologous species γ globulin content of rabbit maternal and fetal serum following the intravenous administration of human and bovine γ gobulin.*

Time of circulation (hours)	Human serum γ globulin administered			Bovine serum γ globulin administered		
	γ globulin titer of maternal serum* following injection†	γ globulin titer of fetal serum*	Number of fetuses studied	γ globulin titer of maternal serum* following injection†	γ globulin titer of fetal serum*	Number of fetuses studied
¼‡	+§	0§	2			
½‡	+§	0§	5			
1	1:1500	0	16	1:1280	0	11
1½‡	+§	0	5			
2	1:500	0	7	1:200	0	7
4	1:100	0	7	1:80	0	16
6	1:100	0	4	1:80	0	3
8	1:80	0	7	1:80	0	6
10	1:80	1:2	12	1:80	+‖	9
12	1:80	1:4	14	1:80	+‖	4
16	+§	+§	2	1:80	+‖	
	1:80	1:4	8			
24	+§	+§	10	1:80	+‖	11
	1:80	1:10	8			
48	1:40	1:10	6	1:40	1:5	3
	1:40	1:10	4			
72	1:40	1:10	12	1:20	1:5	5
96	1:40	1:20	9	1:20	1:5	12
120	1:40	1:40	4	1:5	1:5	7

* Titrated against antiserum with antibody titer 1:640.
† Control precipitin test on maternal serum prior to injection in each instance was negative.
‡ Received only ±50 mg of γ globulin.
§ Positive test on qualitative ring test; titer not done.
‖ Positive test (precipitate noted) on qualitative ring test but a titer beyond 1:1 could not be determined.

next few days. Between 24 and 48 hours the titer dropped to 1:40 and was determined at this level until 120 hours after the initial injection. A similar pattern was observed in those animals that had received bovine γ globulins: The titer at 1 hour was 1:1280, at 2 hours 1:200, at 4 hours 1:80. It remained at this level until 48 hours when the titer was found to be 1:40; at 72 to 96 hours it fell to 1:20 and at 120 hours it was 1:5. The passage of heterologous serum globulin through the placenta was evidently a slow process, and in neither instance did the titer of the placental serum reach that of the maternal serum until after 120 hours of circulation through the maternal blood stream. In those animals that received human γ globulin none appeared in the fetal blood stream until after 10 hours. The titer then rose slowly reaching 1:4 at 12 to 16 hours, 1:10 at 48 to 72 hours, 1:20 at 96 hours, and 1:40 at 120 hours. In those animals receiving bovine γ globulin, this was first found in the fetal serum after 10 hours, but in such small amounts that it could not be detected by specific antiserum when diluted 1:2 by saline. At 48 hours the fetal titer was 1:5 and remained at this level until 120 hours. In those animals which showed an absence of the administered serum γ globulin in the fetal blood serum, the globulin was detected in the placental blood serum in the same or nearly the same titer as that found in the maternal serum.

The placental transmission of homologous antiserum differed from that of heterologous serum γ globulin. The results are summarized in table 2. Ten pregnant rabbits each received 10 ml of rabbit antiserum (antibody titer 1:640) intravenously, 4 animals received antibovine serum γ globulin, 6 received antihuman serum γ globulin. Dilutions of maternal and fetal serum were then studied for antibody content by precipitin tests after allowing the antiserum to circulate for 1, 2, 4, and 8 hours. The findings in both series of animals were essentially similar. Antibodies were found in the fetal serum one hour after

TABLE 2.—*Comparative antibody titers of maternal and fetal serum following the intravenous administration of 10 cc of antiserum to pregnant rabbits.*

Antiserum administered	Time of circulation (hours)	Antibody titer of maternal serum following injection*	Antibody titer of fetal serum	Number of fetuses studied
Rabbit	1	1:16	1:4	7
Antibovine	2	1:32	1:16	4
γ globulin	4	1:16	1:16	10
Antibody titer 1:640	8	1:16	1:16	4
Rabbit	1	1:32	1:8	6
Antihuman	1	1:32	1:32	5
γ globulin	2	1:16	1:4	9
Antibody titer 1:640	4	1:32	1:32	8
	4	1:32	1:32	2
	8	1:32	1:32	3

* Control precipitin test on maternal serum prior to injection in each instance was negative.

administration to the mother, the fetal titer being approximately one-fourth that of the maternal except in one animal in which the titers were equal. After two hours the fetal antibody titer was one-fourth to one-half that of the maternal titer and they were equal at 4 and 8 hours.

DISCUSSION

It is apparent from these results that the passage of serum antibody through the rabbit placenta differs markedly from the transmission of heterologous serum γ globulin. The rapid drop in the heterologous γ globulin content of the maternal serum in the first few hours after its administration is similar to the findings of Dean, Goldsworthy, and Ten Broeck,[22] Dixon, Dammin, and Bukantz,[23] and Knox,[24] and may be attributed to the fact that these globulins meet the fate of foreign proteins within the body of the rabbit undergoing deposition in the reticulo-endothelial system, possibly excretion, etc., whereas when rabbit antiserum was administered intravenously the antibody globulin so introduced acted as a homologous serum protein within the blood stream. The delayed time of appearance of heterologous γ globulin in the fetal blood stream and its slow rate of transmission suggest that mechanisms other than simple diffusion are involved.

There is no reason to believe that this difference is due to the relative strengths of the antiserums employed. The same lot of pooled antiserum was used for the precipitin tests as was administered intravenously in the second series of animals. This antiserum was found capable of detecting specific antigen in concentrations of one part per million and could therefore detect even a minute serum content. It is entirely possible that the heterologous γ globulins were picked up in the fetal reticulo-endothelial system, thereby removing it from the blood stream as it entered into the circulation. However, just as in the adult this should have reached its maximum in 1 to 2 hours and not require 5 days for equalization of concentration on both sides of the placenta. There was no difference noted in the manner of placental transmission of homologous antibodies against bovine

22. Dean, H. R., Goldsworthy, N. E. and Ten Broeck, C. 1930, The rate of disappearance of injected horse serum from the blood of the rabbit. J. Immunol. **18**: 95–103.
23. Dixon, F. J., Dammin, G. J. and Bukantz, S. C. 1950, Effect of sensitization and irradiation on the fate of I^{131} labelled bovine gamma globulin in rabbits and mice. Federation Proc. **9**: 380.
24. Knox, W. C. 1950, I^{131} as antigen label in circulating serum of non-immune rabbits. Federation Proc. **9**: 336.

and human serum γ globulin. This may be expected since rabbit antibodies have been found to have physicochemical and immunological identity.[17,25]

Apart from the obvious difference based on their role as either a foreign protein or homologous serum protein, there are no apparent reasons to account for the difference in placental permeability of the two substances, so that antibody globulin is transmitted rapidly and equalizes itself on both sides of the placenta, whereas heterologous serum γ globulins are delayed in their passage and equilibrium is not reached until the 5th day. Antibodies are considered to be modified serum γ globulins and the physicochemical properties of antibody globulin are very similar or even identical with those of normal serum globulins. Presumptive evidence of this is gained by physical and chemical studies of the normal serum γ globulin fraction and purified antibody. These show apparent similarities based upon (1) electrophoretic mobilities,[8–11,16,17] (2) ultracentrifuge studies, sedimentation and diffusion measurements,[11,16–18] (3) molecular weights,[17] (4) isoelectric points[17] and solubility studies,[16] (5) chemical analyses,[15,16] and (6) immunologic relationships depending upon their antigenic specificity.[16,17] Immune globulins and certain antitoxins, bacterial agglutinins and protective bodies against some viruses were found to be concentrated within the serum γ globulin fraction on separation and immunologic assay.[10,13,19]

On the assumption that antibody globulin is identical with normal serum globulin, perhaps this difference of behavior in passing the placental barrier in the rabbit might be explained on the basis of species specificity and differences between rabbit, bovine, and human serum γ globulin. This is indeed a possibility; however, Nichol and Deutsch[18] have found that γ_2 globulins of various animal species (including human and rabbit) are quite similar as regards their electrophoretic and sedimentation behavior, and from the point of view of physical properties several γ_2 globulins appear to be quite similar to those found in human plasma. Their respective molecular weights are likewise in the same range, rabbit γ globulin having a molecular weight of 160,000;[18] bovine, 171,000;[26] and human, 156,000[10] and 171,000.[12] The nature of any difference existing between antibodies to a given antigen produced in different species has not been correlated with physicochemical or immunological properties.[17]

Despite this, antibodies must differ from other serum globulins in some manner, for they are quite specific immunologically. Boyd[16] feels that antibodies must contain spatial differences since they can distinguish minute chemical or spatial differences, a characteristic not possessed by the other globulins, and that this specificity must reside in some chemical differences in at least part of the molecule.

Although no explanation can be given for the difference in fate of homologous antibody globulin and heterologous serum γ globulins when both present themselves at the placental barrier, certain possibilities are suggested. (1) Though serum γ globulins of different animal species are quite similar in their physicochemical characteristics, especially as regards their electrophoretic

25. Treffers, H. P. and Heidelberger, M. J. 1941, Quantitative experiments with antibodies to a specific precipitate. J. Exper. Med. **73**: 125–140, 293–297.

26. Hess, E. L. and Deutsch, H. F. 1948, Biophysical studies of blood plasma proteins. VIII. Separation and properties of the gamma globulins of the sera of normal cows. J. Am. Chem. Soc. **70**: 84–88.

and sedimentation behavior, yet some minute differences may exist among the serum globulins of different species in their shape or spatial arrangement which may be responsible for determining their placental transmission. (2) Antibodies are physiochemically similar to normal serum γ globulins, yet not identical. Perhaps in the process of synthesis of antibody, one of the modifications that the globulin undergoes may result in a difference in its ability to pass through the placenta.

An analogous situation may not exist in the placental transmission of antibody in the actively immunized animal. Gamma globulin and antibodies have been found within lymphocytes[27,28] suggesting that lymphocytes might be antibody carriers. However, preliminary data obtained in this laboratory working with P^{32}-tagged lymphoid cells seems to suggest that peripheral white blood cells and cells of lymphoid tissue do not penetrate the placental barrier.[29]

It has been suggested that the globulin molecule, like other proteins, does not pass through the placenta intact but is probably synthesized from its constituent amino acids by the fetal tissues.[2] An analogous situation regarding antibody globulin hardly seems likely, for the specificity of antibody action probably depends on their shape, structure, spatial arrangement at their surface, and is possibly due to the specific sequence of amino acids in the peptide chain and/or a specific folding or branching of this chain.[15] It is difficult to imagine that the fetal tissues possess the capacity to reassemble these constituent amino acids in exact duplication of their original arrangement once the antibody has been broken down on the maternal side of the placenta and the amino acids diffused through into the fetal circulation.

Kutner and Ratner[30] have explained differences in placental permeability of antibody on the basis of placental structure. In their experience the human and rabbit along with guinea pig and mouse have a placental partition of only one layer, thus permitting the placental transmission of antibody. This is in contrast to the multiplicity of cell layers separating the maternal and fetal circulations in the cow, sheep, horse, and goat, where the placenta has been found to be impermeable to antibody. Despite Kutner and Ratner's findings of human and rabbit placental similarity, it should be noted that there may be important structural differences in the placentas of these two species. However, the findings in this study are provocative in that if on the assumption that heterologous serum γ globulins are rapidly reduced in the blood stream by the processes of deposition in the reticulo-endothelial system, combining with the antigens, excretion, etc., and if these globulins are transmitted slowly through the placenta resulting in a low fetal serum titer, then the question arises as to how much immunity is conferred upon the fetus when the pregnant female is passively immunized with heterologous species antiserum.

SUMMARY

1. A series of 32 pregnant female rabbits were given human and bovine serum γ globulin and a similar series of 10 rabbits received homologous anti-

27. Dougherty, T. F., Chase, J. H. and White, A. 1945, Demonstration of antibodies in lymphocytes. Proc. Soc. Exper. Biol. & Med. **57**: 295–298.
28. Kass, E. A. 1945, The occurrence of normal serum gamma globulin in human lymphocytes. Science, **101**: 337–338.
29. Cohen, S. G. and Karlson, E. L. Unpublished data.
30. Kutner, A. and Ratner, B. 1923, The importance of colostrum to the newborn infant. Am. J. Dis. Child. **25**: 413–434.

serum intravenously. After varying time intervals precipitin studies were made on the serum of the mother and fetuses removed by Cesarean section.

2. Comparative serum titers determined by precipitin tests indicate a difference in the placental transmission of heterologous serum γ globulin and homologous antibodies in the rabbit. The transmission of human and bovine γ globulin was delayed and these did not appear in the fetal circulation until after 10 hours, and the titer in maternal and fetal serum were not equal until 5 days following administration. Homologous antiserum, on the other hand, passed through the placenta rapidly, was found in the fetal serum in one hour, and was found in equal titer in the circulation on both sides of the placenta at 4 hours.

3. An explanation for the difference in behavior of heterologous serum γ globulin and antibody globulin at the placental barrier is not apparent. Speculation as to the possible reason for this and its implications are discussed.

VITAMIN REQUIREMENTS OF DWARF COLONY VARIANTS OF BACTERIA

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The occasional appearance of tiny variant bacterial colonies on nutrient agar plates has often been observed. The cells composing these colonies were first recognized as having arisen from large-colony-forming cells by Jacobsen[1] and by Fromme,[2] and the term "dwarf" was applied to the small colonies. Hadley[3] in 1931 aroused much interest in the dwarf colonies by postulating that the organisms from them are a stage in the developmental cycle of vegetative cells. He used the adjective "gonidial" in referring to the organisms and the letter "G" to indicate the small colonies produced by such cells. Present investigators (cf. Braun[4]) believe that small-colony-forming cells are discontinuous variants that are constantly arising in bacterial populations.

The variant cells grow quite slowly, and it is possible that they are unable to efficiently synthesize essential growth factors that are absent or present only in trace amounts in nutrient agar. Accordingly, an acceleration of the growth rate resulting in the formation of larger colonies might occur if certain nutrilites in sufficient quantity were to be added to the medium. The experiments described in this paper were performed to determine to what extent such added nutritive factors can influence the cells of small colony variant strains of bacteria to produce large colonies.

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1. Jacobsen, K. A. 1910, Centralbl. f. Bakt., I, Orig. **56:** 208–216.
2. Fromme. 1911, Centralbl. f. Bakt., I, Orig. **58:** 445–453.
3. Hadley, P., Delves, E. and Klimek, J. 1931, J. Infect. Dis. **48:** 1–159.
4. Braun, W. 1947, Bact. Rev. **11:** 75–114.

MATERIALS AND METHODS

Dwarf strains.—Eight stable dwarf-colony-forming strains were isolated from large-colony-forming strains of 4 bacterial species: *Alcaligenes faecalis*, *Micrococcus pyogenes*, *Sarcina lutea*, and *Shigella sonnei*. The method of isolation consisted of inoculating nutrient agar plates containing 1% lithium chloride with cells of large-colony-forming strains that had been aged for 6 weeks in nutrient broth containing 5% peptone. After incubation of the plate cultures for 2 days at 37 C, cells of colonies that had a diameter less than 0.15 mm were subcultured on plain nutrient agar. Subculture of cells of 8 such colonies resulted in the formation of new dwarf colonies on plain nutrient agar. Repeated subculturing on this medium during a 12-month period resulted in no change in the size, texture, or number of hours required for the production of dwarf colonies by the majority of cells of these 8 strains. Occasionally, in the process of keeping the dwarf cultures in stock in the laboratory, a few cells of each strain reacquired the ability to form large colonies on plain nutrient agar.

A 9th dwarf strain was not isolated from a large-colony-forming strain of bacteria but rather appeared as a slow-growing contaminant on a sheep blood agar plate that had been stored for several weeks in the refrigerator.* Cells of this strain possessed morphologic and biochemical characteristics, as well as the ability to grow in a wide temperature range, similar to 4 large-colony-forming animal pathogenic species of the genus *Corynebacterium*. No cells of this strain appeared that could produce large colonies on plain nutrient agar.

Attempts to stimulate large colony formation.—In attempts to stimulate cells of the 9 dwarf

* The author wishes to thank Major Morris D. Schneider, A.U.S., for supplying this strain.

strains to form large colonies, the nutrilites listed in table 1 were added singly and in various combinations aseptically to samples of nutrient agar that were then poured into a petri dishes. After the plates were incubated for sterility, a small loopful of a saline suspension of cells of a particular dwarf strain was streaked on each agar surface. Control plates of plain nutrient agar were included in each experiment. Very dilute suspensions of organisms were used in order to obtain discrete, well-isolated colonies. The streaked plates were maintained at 37 C for at least 9 days with daily measurement of colonial diameter.

TABLE 1.—*Substances added aseptically to enrich samples of nutrient agar.*

Substance	Amounts added to 1 ml of nutrient agar			
Adenine sulfate	200	to 2000		μg
Adenylic acid	0.8	to 160		μg
p-Aminobenzoic acid	0.1	to 200		μg
Biotin	0.1	to 1.0		μg
Calcium pantothenate	0.5	to 100		μg
Co-carboxylase	0.000015	to 72		μg
L-Cystine	5.0	to 500		μg
Diphosphopyridine nucleotide	2.2	to 66		μg
2-Methyl-5-ethoxy-methyl-6-amino pyrimidine	0.001	to 360		μg
4-Methyl-5-β-hydroxyethyl thiazole	0.001	to 360		μg
Niacin	0.3	to 300		μg
Nicotinamide	0.3	to 300		μg
Pteroylglutamic acid	0.1	to 100		μg
Pyridoxine hydrochloride	0.5	to 50		μg
Reticulogen	8×10^{-9}	to 4.4×10^{-4}		ml
Riboflavin	0.5	to 45		μg
Sodium nucleate	100			μg
Thiamin hydrochloride	0.00003	to 330		μg
Tween 80	83	to 3300		μg
Vitamin B_{12}	0.0015	to 0.15		μg
Yeast flakes (Brewers')	3.75	to 5000		μg

EXPERIMENTAL RESULTS

When cells of 4 of the 9 dwarf strains were subcultured on certain types of enriched nutrient agar (see table 2), large colonies were produced on the first transfer. Cells of 2 other strains formed large colonies on veal infusion agar but small colonies on every other type of enriched nutrient agar tested. Cells of the remaining 3 dwarf strains produced large colonies on plain nutrient agar after 5 or 6 days of incubation; no type of enriched or complex medium was found that could accelerate their growth.

Of particular interest was the growth response of cells of dwarf variant strain II of *Alc. faecalis* to nutrient agar enriched with thiamin and vitamin B_{12}; this response is illustrated in figures 1 and 2. All of the well-isolated colonies on each plate had approximately the same diameter. To obtain the large brown colonies after 8 days of incubation, the amount of added thiamin could be as low as 16.5 mμg per ml nutrient agar, or as high as 20,000 times that amount. When the quantity of added thiamin was lower than 16.5 mμg, proportionately smaller final colonies were produced; if no thiamin was added but only an optimal amount of vitamin B_{12} (50 to 150 mμg per ml nutrient agar), only white dwarf colonies were produced.

If, on the other hand, an optimal amount of thiamin was added to nutrient agar but vitamin B_{12} omitted entirely, brown medium-sized colonies were formed after 8 days of incubation. These colonies did not increase in size if additional thiamin was pipetted on the surface of the agar or if incubation was continued as long as 21 days. If at any time during the 21 days, however, an optimal amount of vitamin B_{12} was pipetted on the agar surface, after a few additional days of incubation the colonies increased in size to the dimensions of the large brown colonies previously described.

TABLE 2.—*Mediums on which cells of the 9 dwarf strains formed large colonies.*

Dwarf strain	Type of medium
Alc. faecalis dwarf variant	
strain I	NA* enriched with reticulogen
strain II	NA enriched with reticulogen or with thiamin plus vitamin B_{12}
strain III	Plain NA (5 to 6 days at 37 C)
Corynebacterium sp. dwarf strain	NA enriched with thiamin
Sar. lutea dwarf variant strain	Veal infusion agar (10% of colonies large; 90% small)
M. pyogenes dwarf variant	
strain I	Plain NA (5 to 6 days at 37 C)
strain II	Plain NA (5 to 6 days at 37 C)
strain III	Veal infusion agar
Shig. sonnei dwarf variant strain	NA enriched with yeast flakes (slightly larger colonies)

* Nutrient agar.

The thiazole and pyrimidine components of thiamin when added separately or in combination to nutrient agar were entirely inactive in stimulating large-colony-formation by cells of this dwarf strain. All substances listed in table 1 that do not contain thiamin (except vitamin B_{12}) were also inactive. Co-carboxylase was only as active as an equimolar concentration of thiamin. Subculture of cells of the large or medium-sized brown colonies back to plain nutrient agar resulted in the development of small brown colonies. Subsequent subculture on this medium, accompanied by dilution and wearing out of the thiamin and B_{12} molecules that had been adsorbed on or contained in the cells, resulted in the production of only tiny white colonies.

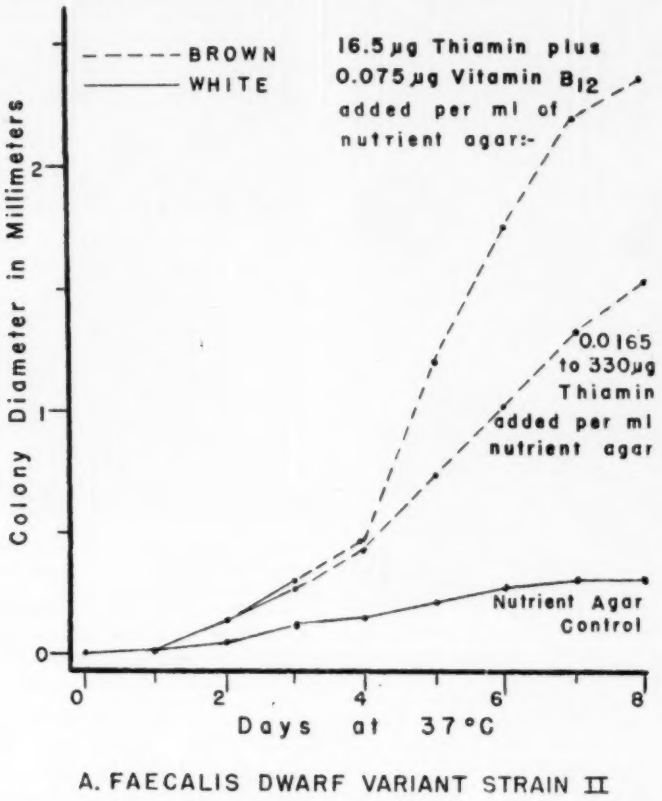

FIG. 1.—The effect of filtered thiamin hydrochloride and autoclaved vitamin B_{12} added to nutrient agar on the size and pigment of well-isolated colonies.

The growth response of cells of the *Corynebacterium* sp. dwarf strain to nutrient agar enriched with thiamin is illustrated in figures 3, 4, and 5. All of the well-isolated colonies on each plate had approximately the same diameter. To obtain the large yellow colonies after 6 days of incubation, the amount of

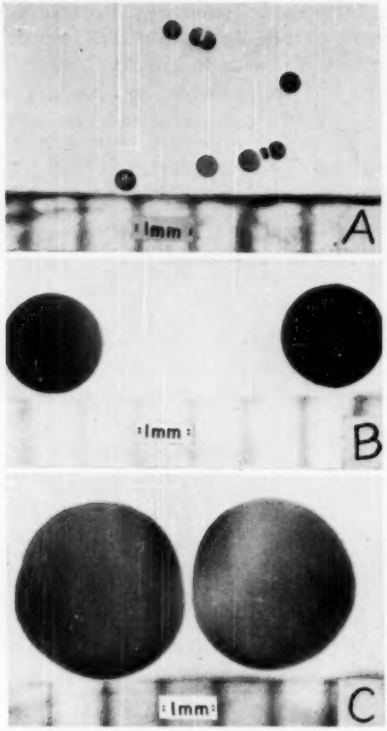

FIG. 2.—Colonies of *Alc. faecalis* dwarf variant strain II; 8 days at 37 C. Space between each 2 adjacent lines of ruler = 1 mm.

A—plain nutrient agar, B—nutrient agar containing 16.5 μg added thiamin per ml, C—nutrient agar containing 16.5 μg added thiamin plus 0.075 μg added vitamin B_{12} per ml.

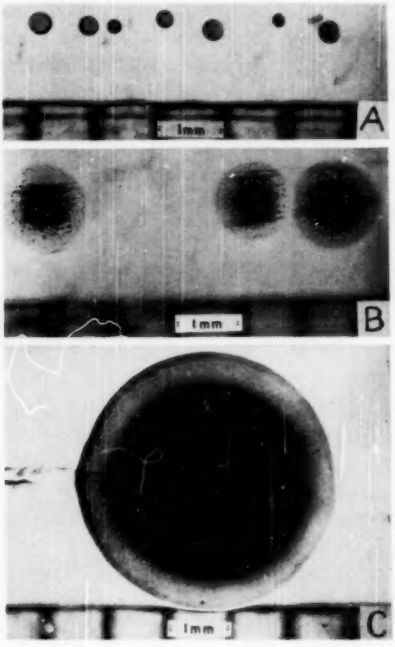

FIG. 5.—Colonies of *Corynebacterium* sp. dwarf strain; 6 days at 37 C. Space between each 2 adjacent lines of ruler = 1 mm.

A—plain nutrient agar, B—nutrient agar containing 0.0006 μg added thiamin per ml, C—nutrient agar containing 1.65 μg added thiamin per ml.

added thiamin could be as low as 3.3 mμg per ml nutrient agar or as high as 100,000 times that amount. When the quantity of added thiamin was lower than 3.3 mμg, proportionately smaller final colonies were produced.

The thiazole and pyrimidine components of thiamin when added separately or in combination to nutrient agar were entirely inactive in stimulating large-colony-formation by cells of this dwarf strain. All substances listed in table 1 that do not contain thiamin (including vitamin B_{12}) were also inactive. Co-carboxylase was only as active as an equimolar concentration of thiamin. Subculture of cells of the large yellow colonies back to plain nutrient agar resulted in the development of small yellow colonies, and subsequent subcultures on this medium yielded only the tiny white colonies.

When suboptimal amounts of thiamin were added to nutrient agar, a linear relationship between amount of thiamin and final colony diameter of the *Corynebacterium* sp. dwarf strain was found to exist between 0.2 and 1.8 mμg of the added vitamin (fig. 4). Accordingly, an unknown amount of

thiamin in a solution could be accurately determined, provided that the solution was sufficiently diluted and the plates to which the solution was added were run simultaneously with control plates containing known amounts of added thiamin.

subcultured on plain nutrient agar, after the first subculture only the tiny colonies characteristic of each strain appeared.

Under the conditions described in table 2, cells of 5 of the 9 dwarf strains produced large colonies identical in

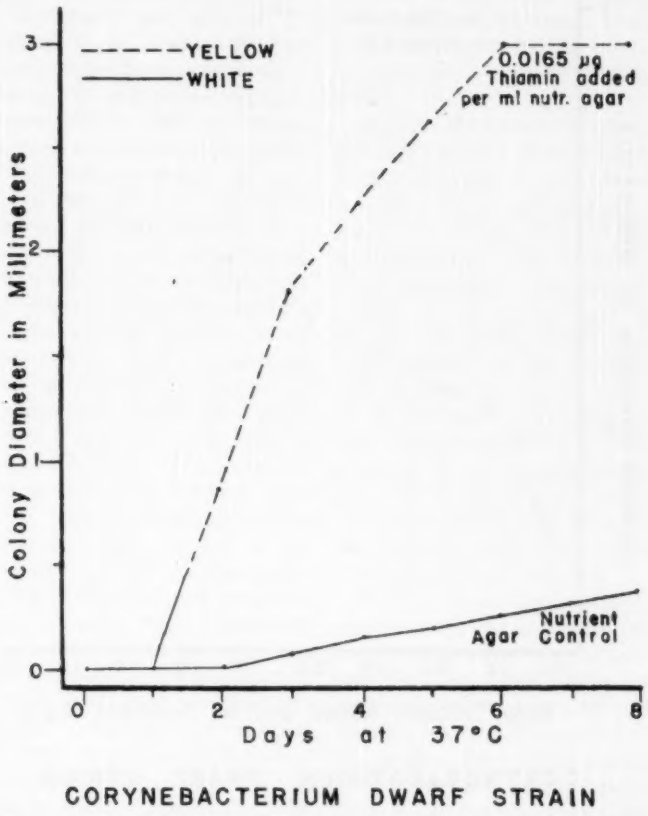

FIG. 3.—The effect of filtered thiamin hydrochloride added to nutrient agar on the size and pigment of well-isolated colonies.

No significant differences in cellular morphology were noted between cells of the tiny colonies of each of the 9 dwarf strains and their respective artificially stimulated large colonies. When cells of the large reverted colonies were again

morphology and pigment to those formed by their respective original parent large-colony-forming cells. Also, the cells of the large reverted and large original colonies of these 5 strains had identical morphologic and biochemical

characteristics. The reverted colonies of three additional dwarf strains (*Alc. faecalis* variant strains I and II and *M. pyogenes* variant strain I) differed from white colonies of the original strains in that the reverted colonies were compared with, a known specific large-colony-forming strain of bacteria.

DISCUSSION

The use of nutrient agar as the basal medium in this study was adopted for

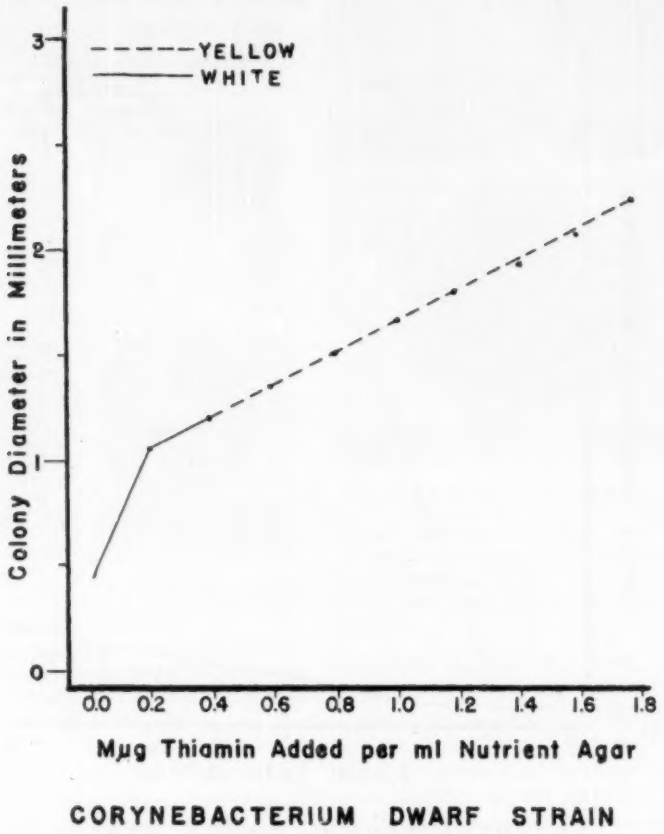

FIG. 4.—The effect of filtered thiamin hydrochloride added to nutrient agar on the size and pigment of well-isolated colonies after 6 days at 37 C.

pigmented. Cells of the large reverted and large original colonies of these three strains were otherwise similar in morphologic and biochemical characteristics. The 9th dwarf strain (*Corynebacterium* sp. dwarf strain) was not derived from, and hence could not be

two reasons. First, it was desired to work with small-colony-forming strains similar to those studied by earlier investigators. Many of the strains reported in earlier work were recognized and characterized because they produced tiny colonies on nutrient agar.

Secondly, if cells of small-colony-forming strains lack the ability to efficiently synthesize a substance needed for rapid growth, it would have been comparatively more difficult to isolate such strains on mediums more complex than nutrient agar. Such mediums might already contain the essential substance(s) preformed, and cells of the dwarf strains would always produce large colonies under these conditions.

Conversely, it might be expected that biochemically deficient small-colony-forming strains could be readily obtained on mediums simpler in composition than plain nutrient agar. Ryan and Schneider[5] and Davis[6] have demonstrated that this is true. The former authors, for example, upon subculturing irradiated cells of *Escherichia coli* on a minimal agar medium that supported large-colony-formation by the original wild type cells, obtained certain microcolonies whose cells would form large colonies when supplied with optimal amounts of histidine. The minimal medium was shown to contain trace amounts of histidine that permitted formation of only tiny colonies by the "histidine-less" variant cells. Similarly, of the 8 dwarf variant strains observed in the present study, 5 are apparently biochemically deficient variants, that is, they readily produce large colonies when provided with preformed essential nutrilites (table 2) that are not required for large-colony-formation by their original parent cells.

Eight of the 9 dwarf strains observed in the present study produced, under conditions listed in table 2, large colonies that were pigmented. The original large-colony-forming strains from which 3 of the dwarf strains were obtained, however, produced only large white colonies on plain or enriched nutrient agar. A somewhat analogous observation has been made on an adenine-requiring mutant of *Neurospora crassa*. When supplied preformed adenine the mutant strain produces a purple pigment. The pigment is believed to be "an abnormal metabolic product from a labile adenine precursor that accumulates as a result of the genetic block in adenine synthesis".[7]

Because the amount of added thiamin necessary for the formation of colonies of a definite size of the *Corynebacterium* sp. dwarf strain remained constant for many months and on every preparation of nutrient agar (Difco) tested, a microbiological method of thiamin assay suggested itself, Advantages in using colonial size of this strain to assay thiamin include: (a) the simplicity of the experimental procedure and the need for no expensive equipment or materials, and (b) the extremely specific growth response of the organisms to a small amount of added thiamin and their formation of only tiny white colonies on all types of enriched nutrient agar not containing added thiamin.

The principal disadvantage in using organisms of this strain for thiamin assay is their slow rate of growth. To eliminate this disadvantage, stock pour plates of plain nutrient agar containing the tiny white colonies can be prepared in advance and stored at 4 C until needed for an actual assay determination. By the use of assay disc techniques (Pontecorvo[8]) end points can then be obtained within the subsequent incubation period of 24 to 48 hours.

A dwarf strain such as this is also useful in enabling students in introduc-

5. Ryan, F. J. and Schneider, L. K. 1949, Genetics, **34**: 72–91.
6. Davis, B. D. 1949, Arch. Biochem. **20**: 166–167.
7. Mitchell, H. K. and Houlahan, M. B. 1946, Federation Proc. **5**: 370–375.
8. Pontecorvo, G. 1949, J. Gen. Microbiology, **3**: 122–126.

tory courses in bacteriology to demonstrate for themselves the fact that bacteria may need vitamins for adequate growth. The tools and techniques required for a simple experiment of this nature are readily available in such introductory courses.

SUMMARY

Nine strains of bacteria that formed tiny colonies on plain nutrient agar were subcultured on nutrient agar enriched with various growth factors. The factors that were effective in stimulating cells of these dwarf strains to produce large colonies were: thiamin (*Corynebacterium* sp. dwarf strain), thiamin plus vitamin B_{12} (*Alcaligenes faecalis* dwarf variant strain II), reticulogen (*Alc. faecalis* dwarf variant strain I), and yeast extract (*Shigella sonnei* dwarf variant strain). Veal infusion agar was effective in stimulating large-colony-formation by cells of two other dwarf strains. Cells of the remaining three dwarf strains slowly produced large colonies equally well on plain or enriched nutrient agar or on veal infusion agar.

Cells of the reverted large colonies of all of the dwarf strains formed only tiny colonies when again subcultured on plain nutrient agar. Eight of the 9 dwarf strains were definite variant strains derived from large-colony-forming bacterial populations. Cells of 5 of these strains, whose growth was stimulated on enriched mediums, are considered to be biochemically deficient variants.

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